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PLANT PHYSIOLOGY

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INVESTIGATIONS ON NITROGEN COMPOUNDS AND NITROGEN METABOLISM IN PLANTS. II. VARIABLES IN TWO-DIRECTIONAL PAPER CHROMATOGRAPHY OF NITROGEN COMPOUNDS: A QUANTITATIVE PROCEDURE

JOHN F. THOMPSON[1] AND F. C. STEWARD[1]

(WITH TWO FIGURES)

Received October 24, 1950

In Part I (**21**), the conditions under which the ninhydrin reaction on paper may be carried out quantitatively and applied to 2-directional paper chromatograms have been defined.

In this part (II) of the investigation, the variables operating in the chromatography will be considered. It will be shown that when these variables are brought under control, the ninhydrin reaction on paper may be applied in such a way as to permit the quantitative determination of a large number of amino acids on a single chromatogram sheet.

Hitherto, workers who have concerned themselves with quantitative paper chromatography of amino acids have concentrated mainly on the means to be adopted for determining the amino acids after chromatography, whether by ninhydrin or otherwise. (See Part I, refs. **5–14.**) Also, most of the quantitative procedures hitherto described have utilized only the 1-directional, or "strip," chromatograms. (See Part I, refs. **7, 8, 9, 11, 12, 13.**) Unless the selectivity of the "2-directional" procedures is exploited in the quantitative method, chromatographic methods would be inapplicable to most biological problems. No strip or 1-directional method can readily reveal the great variety of amino compounds known to be present in plants or in protein hydrolysates.

The objective was a method by which the amino acids could first be separated on paper and then quantitatively determined. This method requires not only clean-cut, but reproducible, separations on paper of as many amino compounds as possible, but also that the quantitative recovery should be reproducible and preferably complete to avoid the necessity of calibrating

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the procedure on every occasion that it is used. By use of a sufficient number of simultaneous standards, it is comparatively simple to obtain quantitative results on an unknown sample as POLSON (**18**), in fact, has done. This method is, however, laborious and expensive in the lavish use of standards per sample analyzed. Furthermore, in such a purely relative method, the variables operating remain both unidentified and uncontrolled and the possibility remains that unrecognized factors may produce unexpected errors.

BLOCK (**3**) has reported a method whereby amino acids are determined quantitatively by colorimetric means, but has not tried particularly to control variables in the development of color although an anaerobic atmosphere during chromatography was used. To obtain acceptable values, a considerable number of chromatograms (25) was necessary. This method is, of course, not feasible for routine work.[2] In the work to be described, the variables have been controlled in such a way that quantitative results may be obtained without preparing standard curves for each amino acid on each occasion when samples are analyzed.

Separation of amino compounds on paper by 2-dimensional paper chromatography

The basic procedures involved, following CONSDEN, GORDON, and MARTIN (**4**), are well known. The type of apparatus we have used has been briefly described (**19**). For quantitative work, we make use only of a 2-directional procedure based on the use first, of a phenol-water moving phase and second, of a collidine-lutidine-water phase moving in a direction at right angles to the phenol direction. In order to achieve, consistently, the degree of separation which is necessary, the following points require special attention.

The preferred paper sheets are Whatman no. 1 (18½ by 22½ inches). Ten other types of paper have been examined, mainly in the attempt to find one which would cope with larger amounts of amino acids. Briefly, no other paper was preferable for routine work. A particular grade of paper (Schleicher and Schuell, no. 112) has merit when very compact and discrete spots are required: no papers which are watermarked can be recommended for 2-directional quantitative work. KOWKABANY and CASSIDY (**15**) have tested the behavior of 75 filter papers toward five solvents used in chromatography and recommended certain of these for use.

In this study, solvents other than those mentioned below have not been tested. In 2-directional amino acid chromatography, phenol is most used as one solvent. Though there is a wider choice of a second solvent, a collidine-lutidine mixture satisfactorily separates amino acids so that reproducible results may be obtained. Other solvents such as butanol-acetic acid

[2] Later BLOCK (**3a**) elaborated and extended his earlier methods for analyzing protein. He has now discussed the hydrolysis of sample, choice of paper and solvents, development of the color (including special tests for specific amino acids), measurement of the color density, and quantitative estimation of amino acids.

(14) and acetone (1) have been recommended for qualitative work. There is no apparent reason why these should not be satisfactory in quantitative work.

In the use of phenol-water and collidine-lutidine-water as the moving phases of a 2-directional system, the following points should be noted.

The phenol used (Mallinckrodt's) gives a water clear and colorless solution. Reagent grade phenol is adequate: redistilled phenol is not necessary. When used, the phenol is saturated with water at the temperature to be used in the chromatography.

DENT, STEPKA, and STEWARD (9) realized that the "collidine" of CONSDEN, GORDON, and MARTIN (4) and in fact, as often sold, was not pure and contained some lutidine. Consistent results can only be obtained by the use of pure solvents, recognizing that the amino acids move at different rates in the collidine (2,4,6-trimethyl pyridine) and the lutidine (2,4-dimethyl pyridine). In lutidine, the amino acids move too rapidly; in collidine they move too slowly, for most purposes. Therefore, by the use of mixtures of these solvents appropriate rates of movement may be obtained. For ordinary purposes, a 1 to 3 mixture by volume of collidine-lutidine is best. These solvents are purchased in bulk (Koppers Company, Pittsburgh, Pennsylvania) and redistilled, retaining collidine between 168 and 175° C and lutidine between 155 and 162° C. Again, the solvent mixture is saturated with water at the temperature of chromatography and is stored ready for use in this form.

All the early work on paper chromatography of amino acids made use of ammonia and cyanide vapor in the cabinets during the phenol stage. The purpose here is twofold.

(1) The ammonia keeps the moving solvent more alkaline, rendering the basic amino acids relatively more mobile and the acidic amino acids relatively less mobile.

(2) The cyanide vapor retards oxidation of the phenol under these more alkaline conditions, by virtue of its effect on heavy metals.

In our view, the disadvantages inherent in the use of ammonia in the cabinets outweigh its advantages. First, it causes darkening of the papers, even with the use of cyanide, and this becomes important when colorimetric quantitative work is attempted. Conditions which oxidize phenol during chromatography may be prone to deaminate amino acids by interaction with phenol oxidation products. By interaction between ammonia and reactive constituents of the extracts (*e.g.*, keto acids), there is some danger that ninhydrin-reactive substances may be formed as artifacts: this also applies to combination with cyanide if strong reducing agents are present. Therefore, in our work we have abandoned the use of these substances in the cabinets.

The only consequence is that the position on the papers of certain amino acids needs to be re-established for these conditions. Most of the important acids that may be resolved in the presence of ammonia can be resolved without its aid. Histidine does not form a well recognizable spot in the

absence of ammonia. As a further consequence of eliminating ammonia, effects due to the acidity of the phenol-water phase (pH 1 to 2 resulting from acid impurities in phenol) are encountered. To control this, the phenol is adjusted by sodium hydroxide to pH 5 to 5.5 or distilled before use. The principal effect of acidity during the phenol phase of chromatography is that the acidic amino acids tend to spread in the phenol direction, *i.e.*, they streak. This is particularly true of glutamic acid which interferes with glycine and asparagine. It is known that the alkalinity (due to salts) in the papers as manufactured tends to counteract this effect. Removal of these salts from papers, by prior washing with water, tends to accentuate the streaking of these acids when phenol is used (later reference will be made to the use of washed papers).

The usual temperature for chromatography is between 25 and 27° C. Other temperatures, if constant, are suitable, but in the use of solvents saturated with water, the limitations are as follows. At lower temperatures the amino acids move more rapidly in the collidine-lutidine phase (table I)

TABLE I

CHROMATOGRAPHY OF AMINO ACIDS ON PAPER. EFFECT OF TEMPERATURE ON THE MOBILITY OF AMINO ACIDS DURING CHROMATOGRAPHY ON PAPER IN COLLIDINE-LUTIDINE SATURATED WITH WATER. AMINO ACIDS WERE LOCATED ON THE PAPER BY NINHYDRIN, THE DISTANCE OF MOVEMENT WAS MEASURED FROM THE CENTER OF THE INITIAL SPOT TO THE CENTER OF FINAL SPOT.

Temperature	R_f* Value			
	Glutamic acid	Threonine	Alanine	Leucine
15° C	.312	.435	.448	.630
25° C	.208	.287	.262	.505
34° C	.113	.216	.191	.473

* $R_f = \frac{\text{distance of amino acid movement}}{\text{distance of solvent movement}}$

because the moving solvent then holds more water. Since the effect of temperature on the solubility of water in phenol is small, the effect of temperature is not marked on the rates of movement in the phenol phase.

The presence of other water-soluble compounds in the extract analyzed has an adverse effect. In low concentration, the rate of movement of amino acids is reduced. In higher concentration, water-soluble substances, especially salts, bind water so tenaciously that the paper becomes "water-logged" and the amino acids "streak" rather than form discrete spots. Usually, the free amino acids of plants are extracted in cold 75% alcohol; and the extract so obtained is relatively rich in soluble nitrogen compared to its salt or sugar content. This extract is normally concentrated by evaporation *in vacuo* at low temperature (not above 40° C if glutamine is to be preserved) to dryness and is redissolved in water to obtain a solution containing the requisite amount of soluble nitrogen, *i.e.*, about 1 μg. of amino-N per μ-liter. Trichloroacetic acid, or similar solvents for nitrogen

compounds, are not as suitable as alcohol because of the difficulty of removing, or neutralizing, the solvent without adding excessive amounts of salt. In the analysis of proteins by hydrolysis one should, therefore, use hydrochloric acid which may be removed by evaporation.

The sulphur-containing amino acids (methionine, cystine, and their related compounds) present a special problem, partly because of the position they occupy on the chromatograms (*e.g.*, methionine and its sulphoxide which may overlap leucine and γ-amino butyric acid respectively) and partly because of the relative insensitivity of their ninhydrin reactions (*e.g.*, cystine). Because of the slight solubility of cystine it should not represent more than 1% of the total nitrogen in aliquots of 1 μg. N per μl. To overcome these difficulties, the amino acid extract is oxidized with hydrogen peroxide, which may be added to the sample when it is applied to the papers (to ensure complete oxidation an addition of a trace of molybdate may be necessary). The oxidation converts methionine and its sulphoxide to methionine sulphone and cystine to cysteic acid—each of which occupy distinctive positions and react well with ninhydrin.

Variables which affect the quantitative recovery of amino-compounds after 2-directional paper chromatography

Using the quantitative procedure for the ninhydrin reaction on paper (**21**) and 2-directional paper chromatography, the ninhydrin value of the amino acid finally recovered is definitely less than if the amino acid is merely placed on a filter paper, but not chromatographed.

Possible ways in which amino acids may be lost during paper chromatography are as follows: (1) By decomposition (deamination) due to interaction with the solvents or the atmosphere. (2) By adsorption on the paper along the length of travel.

Losses in either of these two categories may be determined by different factors, *e.g.*, time of contact with the solvent, distance of movement on the paper, the degree of saturation of the cabinet, which will determine the water relations of the paper, the presence of oxygen in the cabinets, the pH of the phenol, and the presence on the paper of extraneous solutes (salts or traces of heavy metals).

Since good qualitative separation of the amino acids on the paper is obtained when they are in equilibrium with a water-saturated atmosphere, these conditions have been used throughout. To ensure uniformity, the water content of the papers has been standardized before use in chromatography. The dry paper, with the sample already applied, is placed in a cabinet and allowed to equilibrate to the atmosphere, saturated with water and solvent, for 12 hours before the chromatography begins. Such devices as additional papers kept wet with the solvents become desirable if few papers are used in large cabinets. Thus, the humidity of the cabinets has arbitrarily been controlled at 100% saturation though the degree of saturation is not critical. (See table II.)

TABLE II

LOSS OF AMINO ACIDS ON FILTER PAPER. THE EFFECT OF DEGREE OF SATURATION OF THE PAPERS ON RECOVERY OF AMINO ACIDS AFTER CHROMATOGRAPHY. FORTY MICROGRAMS OF EACH AMINO ACID WAS CHROMATOGRAPHED UNTIL THE SOLVENT FRONT HAD RUN 48 cms.

Amino acid	Percentage recovery of amino acid after chromatography			
	In water-saturated phenol		In water-saturated 1 : 3 collidine-lutidine	
	Saturated papers*	Unsaturated papers**	Saturated papers*	Unsaturated papers**
Glutamic acid	88	88	81	87
Threonine	84	84	96	90
Alanine	85	87	98	98
Serine	90	88	96	92
Valine	....	....	100	96
Leucine	84	92	100	97

*Saturated means that the papers were allowed to equilibrate 48 hours in an atmosphere exposed to a surface of a mixture of the organic solvent and water on the bottom of the cabinet before the addition of the chromatographing solvent.

**Unsaturated means that the chromatographing solvent was added to the cabinet immediately after inserting the dry paper sheets.

The effect of time of contact with the solvent was investigated for samples of amino acid which were stationary on the paper. The length of time was comparable to that normally used in chromatography. The amino acids were analyzed by the method already described (**21**). Briefly, the losses merely due to contact with phenol were slight, those due to collidine-lutidine were nil (table III). Therefore, the losses sustained during chromatography are due to other factors than mere contact with the solvents in air.

Even the slight losses sustained due to mere contact between amino acids

TABLE III

LOSSES OF AMINO ACIDS ON FILTER PAPER. THE EFFECT OF THE PRESENCE OF CHROMATOGRAPHIC SOLVENTS IN CONTACT WITH AMINO ACIDS ON PAPER, IN AIR AND IN AN ATMOSPHERE OF CARBON DIOXIDE.*

Amino acid	Percentage recovery of amino acid in contact with solvent after 24 hrs. at 25° C			
	Phenol saturated with water		Collidine-lutidine 1 : 3 saturated with water	
	Aerobic	Anaerobic	Aerobic	Anaerobic
Glutamic acid	95	100	100	100
Threonine	97	100	100	100
Alanine	98	100	100	100
Leucine	94	100	100	100

*Forty micrograms of each amino acid were placed on a small filter paper square (4 by 4 cm.) which was sprayed with solvent and maintained in a saturated atmosphere for 24 hours at 25° C. Solvents were evaporated at room temperature in moving air. Filter paper was cut up and color was developed by the method of LANDUA and AWAPARA (**16**).

on the paper and phenol-water are eliminated if oxygen is excluded (table III). However, the benefits which accrue from anaerobic conditions during the chromatography are not enough to justify its regular use (*e.g.*, by maintaining a carbon dioxide atmosphere in the cabinets).

The distance of travel of amino acids over the paper is the principal factor in the losses which are sustained during paper chromatography. Figure 1 shows, for different solvents, the relationship between distance of travel and quantity of amino acid measured for two amino acids, valine and

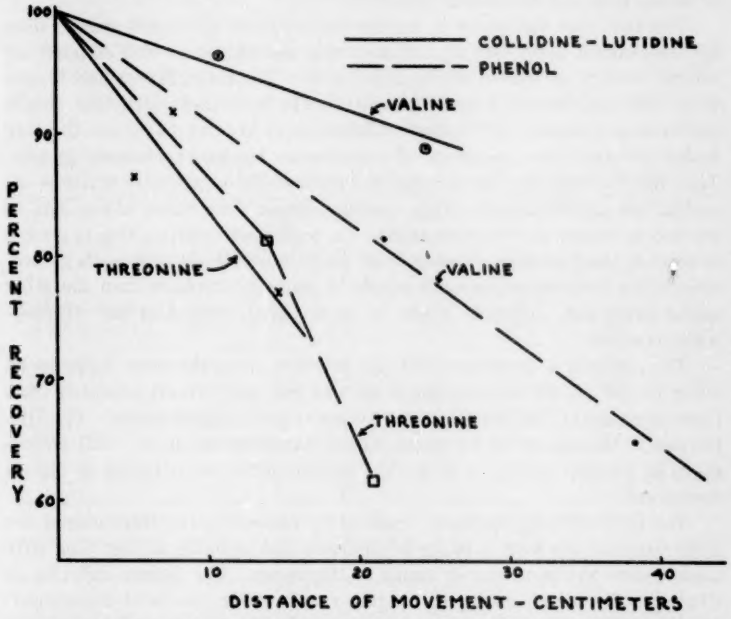

FIG. 1. Chromatography of amino acids on paper: the effect of distance of movement on losses of valine and threonine during chromatography in either phenol saturated with water or in collidine-lutidine saturated with water. Distance measured from initial spot to center of final spot.

threonine. For valine, the losses observed are mainly due to distance of travel, but more loss occurs in the phenol. The results with valine are typical of those for other amino acids, like aspartic and glutamic acids, glycine, alanine, γ-amino butyric acid, the leucines, and the aromatic amino acids. The results for threonine are similar to those for serine and in these cases the losses in the two solvents are similar and greater than those for valine and other amino acids.

In all cases, there is some loss which is roughly proportional to the dis-

tance of travel. Where some loss occurs, irrespective of distance (*e.g.*, in phenol), interaction with the solvent is presumably involved: such losses are reduced under anaerobic conditions (table III) and increased with more alkaline phenol. Therefore, it is imperative in quantitative work to control closely the distance of movement in both directions.

That there is some relation between distance of movement and loss of glycine has been reported by WOIWOD (**23**). Claiming that glycine is atypical, FOWDEN and PENNY (**11**), however, showed no relationship between loss of amino acid and distance of travel.

The fact that the losses of hydroxyamino acids are much greater than for other amino acids (fig. 1) indicates that adsorption as well as partition are involved in movement of the amino acids. Recently, HANES and ISHERWOOD (**13**) and BENTLEY and WHITEHEAD (**1**) have postulated that simple partition as proposed by CONSDEN, GORDON, and MARTIN (**4**) is not the only factor involved in separation of compounds by paper chromatography. They believe that there is an organized water-cellulose complex which is not mobile but tightly bound. This complex, rather than water alone, acts as the second phase in chromatography. A hypothesis such as this is needed to explain the partition obtained with water-miscible solvents such as acetone. The hydroxyamino acids would be more hydrophilic than the other amino acids and, therefore, would be more tightly bound in the cellulose-water complex.

The problem is to ensure that the solvents move the same distance on every paper. Differences in rate of solvent movement result primarily from three causes: (1) Differences in the thickness of the paper sheets. (2) Differences in the amount of water-soluble substances on the paper. (3) Differences in vertical height to which the solvent must move before it travels downward.

The first difficulty has been resolved by measuring the thickness of the filter paper sheets with a paper micrometer and utilizing at one time only those sheets within a narrow range of thickness. The second difficulty is eliminated by prior washing of the paper with water (see later discussion). The third difficulty may be largely removed by the use of accurately made troughs (**19**) or by the use of the ascending method which does not, however, lend itself to such wide separation of spots.

The distance of travel will vary somewhat depending on the amount of water-soluble compounds in the sample. It is, therefore, desirable to reduce to a minimum the losses of amino acids during their travel. A number of factors which might affect the losses in transit on the paper have been studied. These include saturation of the paper and atmosphere with chromatographing solvents, pH of paper or materials impregnated on it, purity of solvents and impurities in the paper.

With phenol, the effect of saturation is not great (table II): with collidine-lutidine, the effect of the degree of saturation is somewhat larger.

Oxidation products of phenol will oxidatively deaminate amino acids and

phenol is more rapidly oxidized, the more alkaline the reaction. If the pH of the phenol is raised above pH 6, the losses of amino acids are increased.

Table III shows that by the elimination of oxygen, the losses of amino acids due to contact with phenol are nil. For this reason, chromatography under anaerobic conditions was tried but the effects were not clear cut. Apparently there are other and greater causes of the loss of amino acids than those due to oxygen.

Since heavy metals catalyze the oxidation of phenol, phenol was purified by vacuum distillation. Chromatography with distilled phenol did not appreciably reduce the observed losses.

By far the most important factor which caused losses during chromatography are water-soluble impurities present on the paper. (See table IV.) Table IV shows that the losses during chromatography on washed papers

TABLE IV

CHROMATOGRAPHY OF AMINO ACIDS ON PAPER: THE EFFECT OF WATER-SOLUBLE SUBSTANCES ON THE PAPER ON THE LOSS OF AMINO ACIDS BY ADSORPTION.*

Solvent	Treatment of paper	Percentage loss of amino acid per cm. of travel				
		Glutamic acid	Threonine	Alanine	Leucine	Serine
Collidine-lutidine	None	2.05	1.81	.41	.57	1.42
Collidine-lutidine	Acid washed	.55	.90	.00	.099	.72
Phenol	None	1.78	1.43	.39	.67	1.07
Phenol	Acid washed	.37	.62	.084	.30	.42
Phenol	Water washed	.41	.71	.18	.67	.55

* Forty micrograms were chromatographed in one direction with either phenol saturated with water or collidine-lutidine (1 : 3) saturated with water. Distance of movement was measured from center of initial spot to center of final spot. Amino acids were determined by the method described in Part I (**21**). Acid-washed papers were washed with 0.1 *N* acid (HCl) and then distilled water until acid free. Water-washed papers were washed thoroughly with distilled water.

are much less than on unwashed papers. Washing of the paper with acid has only a slight advantage over washing with water. However, acid washing has another effect in preventing "bearding" (*i.e.*, formation of a streak in front of the main spot (**15**)). The amount of bearding varies considerably with different batches of paper. Acid (0.3 *N* HCl) removes a water-insoluble substance from the paper which causes bearding. Bearding is undesirable for it causes overlapping of the spots and decreases separation. Acid washing may remove heavy metals (**13**) or peptides (**25**). The substances that cause bearding are apparently not heavy metals because washing the papers with 8-hydroxyquinoline in alcohol does not eliminate this.

Consequently, acid-washed papers should preferably be used in quantitative work, but there are additional effects to be mentioned. Once the paper is washed, the rate of movement of the solvent is greatly increased.

Phenol moves at a rate of about 2.2 cm./hour (25° C) on unwashed papers and about 3.3 cm./hour on the washed. The rate of solvent movement varies somewhat with the thickness of the filter paper. The rate of movement should be rapid because it leaves less time of contact of amino acids with solvent for oxidative destruction. (See table III.) The materials which reduce the rate of solvent movement are water soluble but probably not salts, since additions of salts to the paper (*vide infra*) does not have this effect.

Acid washing of the paper leaves the sheets with residual acidity which cannot be removed with water. This residual acidity causes glutamic and aspartic acids to move as the free acids in phenol, rather than as salts, and to interfere with other amino acids. Neutralizing the acidity of the paper with base and removal of the excess base does not completely solve this problem. Apparently, since the paper is unbuffered, part of the salt of glutamic acid is converted to the free acid by the slight acidity of the phenol causing it to streak. Some of the water-soluble materials which are removed from the paper have an alkaline reaction and they prevent aspartic and glutamic acid, on unwashed paper, from streaking.

To obtain satisfactory separations of the amino acids, washed paper sheets have been impregnated with sodium phosphate buffer of varying pH and concentration and with other salts of varying concentration (NaCl, $CaCl_2$, $BaCl_2$, $MgCl_2$, $Mg(NO_3)_2$, $Mg_3(PO_4)_2$, $CaSO_4$, and $MgSO_4$). Concentrations of salts and buffer used were between 0.025 and 0.25%. Sodium chloride has no obvious effects. Salts of Ca, Ba and Mg have an effect in preventing streaking of aspartic and glutamic acids. (See Foreman's method (**10**) for their precipitation and its modification by DAKIN (**7**).) However, both Ca and Ba salts interfere with the ninhydrin reaction causing a different (red) color. In presence of magnesium salts the basic amino acids move into positions where they overlap the amides (glutamine and asparagine). Magnesium salts are useful, however, in preventing streaking of glutamic and aspartic acids in protein hydrolysates.

The use of papers impregnated with sodium phosphate buffer (0.1%) pH 7.0 to 7.5 is best for overall separation of amino compounds. Higher concentrations of buffer tend to cause overlapping of the glutamic acid and serine spots and of glutamine and alanine as well as to increase losses of amino acids during chromatography. Lower pH of buffer causes glutamic acid to overlap asparagine, and histidine to overlap alanine (with unwashed papers histidine does not even give a well defined spot in the absence of ammonia). At higher pH's, the recovery of amino acids is reduced and lysine and arginine separate into two spots during collidine chromatography, presumably due to separation of the free acid and its salt.

Acid is added to the collidine (pH 8.5) because it causes better separation of certain amino acids (glutamic acid and serine; glutamine and alanine). The fact that the acidic and basic amino acids do move to different positions on a chromatogram depending on the pH conditions and may

even form two spots on the same paper, makes it necessary to be cautious in claiming new amino compounds (STEWARD and THOMPSON, **20**).

As already indicated (p. 425), the sulphur-containing amino acids do not form separate or distinguishable spots until after oxidation with hydrogen peroxide so that methionine is in the form of its sulphone and cystine is in the form of cysteic acid. After washing the paper and impregnating it with 0.1% phosphate buffer, it has been possible to obtain separate and distinct spots for cysteic acid, aspartic acid, glutamic acid, serine, glycine or asparagine, threonine, alanine, histidine or hydroxyproline, lysine, arginine, γ-amino butyric acid, glutamine, proline, methionine sulphoxide, methionine sulphone, valine, the leucines, phenylalanine, tryptophan and tyrosin. Glycine and asparagine, however, overlap so that they cannot be separately measured on the same chromatogram. In the examination of free amino acids of plants, however, glycine is rarely abundant whereas asparagine may be present in relatively large amounts. In acid hydrolysates asparagine does not occur. It is possible to determine glycine in the presence of asparagine since asparagine forms a yellow-brown compound which absorbs only very slightly at 570 mμ (**21**, see fig. 7). Alternatively, asparagine may be hydrolyzed to aspartic acid before chromatography. The determination of asparagine in the presence of large quantities of glycine is more difficult but it can be done by measuring the increase of aspartic acid after hydrolysis.

Hydroxyproline and histidine overlap. However, hydroxyproline has rarely been found in plants in amounts large enough to measure by these means. It takes a relatively large amount of histidine to obtain enough ninhydrin color for accurate measurement. Consequently, histidine may be better determined by other reagents (*e.g.*, use of diazotized sulphanilic acid (**3, 8**)). Leucine and isoleucine do not separate under the conditions described. Other solvents, *e.g.*, butanol, benzyl alcohol or tertiary amyl alcohol (**4, 6, 8, 24**), should be utilized to separate leucine and isoleucine.

It is possible, therefore, to obtain under the conditions described measurements on most of the natural amino acids, found free and in proteins, except for histidine and hydroxyproline. The leucines are commonly determined together.

The standardized chromatographic procedure

THE PREPARATION OF THE SAMPLE.—If non-protein nitrogen compounds are to be measured, the preferred method is to extract the material thoroughly with 75 to 80% alcohol. The solution is evaporated to dryness. The evaporation should be done under reduced pressure at a temperature below 40° C if glutamine is to be determined. Hydrolysis of the proteins is accomplished with 3 *N* HCl (20 ml. per gram of sample) at 120° C for 20 hours. The excess HCl is removed by repeated vacuum distillation or with silver oxide. The solution is adjusted to pH 6.0, if necessary, with sodium hydroxide. The final volume is controlled so that the concentration of amino nitrogen is about 1 to 2 mg. per ml.

Water-soluble materials (salts, sugars, non-nitrogenous organic acids, etc.) in great excess may interfere with the chromatography. Salts can be separated from the amino acids by electrodialysis (**5**); ion exchange techniques may be used to separate the amino acids from sugars.

PREPARATION OF FILTER PAPER.—Whatman no. 1 filter paper (18¼ by 22½ inches) is washed thoroughly (approximately 200 ml./sheet) with 0.3 *N* HCl and enough 0.5 *N* NaOH is put on the paper to neutralize the residual acid. Then the paper is washed thoroughly with distilled water until there is no test for free base. The sheets are treated with 0.1% sodium phosphate buffer at pH 7.0 to 7.5 and dried. The buffer should be evenly distributed without local excesses to cause waterlogging. For small numbers of papers it is convenient to do all this by the same type of operations as in chromatography: for large numbers, large Buchner type funnels could be used (**13**).

APPLICATION OF SAMPLE TO PAPER.—For complete analysis of the amino acids in a sample, it is convenient to chromatograph the sample in three ways. First, a small sample (30 to 50 μg. N) is chromatographed so that satisfactory separation can be obtained between aspartic acid and glutamic acid, serine and glycine, and threonine and alanine. These are acids often found in large amounts so that a small aliquot is necessary to ensure good separation. If one of the amino acids of these three pair is in great excess compared to the other (10 times), measurement of the one present in smaller quantity will be less accurate.

Second, a much larger aliquot (five to 10 times) of the sample is chromatographed. This is treated with 20 μl. of 0.02 M $(NH_4)_2MoO_4$ and 100 μl. of hydrogen peroxide (30%) to oxidize methionine and its sulphoxide to the sulphone and cystine to cysteic acid. The large aliquot is necessary because the sulphur-containing amino acids are usually a small percentage of the total amino acids.

Third, an aliquot of about the same size as the previous one is chromatographed without any hydrogen peroxide treatment. This aliquot is to determine amino acids which may occur in small quantities and may be destroyed by the hydrogen peroxide treatment. This aliquot is also useful for proline which, though often occurring in large quantities, reacts with ninhydrin in a different way than the α-amino acids, and the error is greater in measuring small quantities.

The sample is applied to the corner of a sheet of paper at a point 8 cm. from each side of the paper and is confined to a circle 2 cm. in diameter (about 20 μl. of solution may be applied in one operation to Whatman no. 1 paper). During these operations it is convenient to support the paper around the initial spot with a watch glass. It is preferable to confine the spot to a circle 1 cm. in diameter if those amino acids which tend to overlap are to be determined. In applying the sample to the paper, heat may be supplied as with a Mazda lamp but only as long as free solvent remains.

Two methods may be used for samples to be oxidized. The sample is applied to the paper, then 20 μl. of 0.02 M ammonium molybdate is added,

followed by 100 μl. of 30% hydrogen peroxide, in 20 μl. portions, allowing the peroxide to dry (no heat) after each addition. Another method is to mix the sample, the ammonium molybdate and hydrogen peroxide in a watch glass. This mixture is allowed to dry and the residue is redissolved in water and transferred to the paper.

After the sample has been applied to the sheet, it is allowed to dry. Those samples which have been treated with hydrogen peroxide should be allowed to dry eight hours at room temperature. If the hydrogen peroxide is not thoroughly removed, it will evaporate in the phenol chamber and cause increased oxidation of phenol and consequent destruction of amino acids.

DISPLACED INTERNAL STANDARD.—As a further check on the reproducibility of different chromatograms an internal standard has been used. The purpose of the internal standard is to reveal any marked deviation from the otherwise standard conditions; it may be dispensed with when complete standardization is achieved. A constant amount of a selected amino acid (alanine) is so placed on each sheet that after chromatography it comes to a position on the paper which is not occupied by other amino acids. For this purpose, 40 or 60 μg. of alanine are applied in a displaced position 16 cm. from the long (22½ inches) edge and 8 cm. from the short edge of the paper. After chromatography, this internal standard is in a position not occupied by ordinary amino acids. It cannot be used in this position with hydrogen-peroxide treated samples because the displaced alanine standard occupies a position overlapping that of methionine sulphone.

CHROMATOGRAPHY OF SAMPLES.—A 2-cm. fold, parallel to the shorter side of the paper (18 inches) ensures that the sample lies just beyond the supporting rod when the paper is placed in the trough: for further details see (19). A cabinet 16 inches in width can accommodate four or five troughs with two sheets to each trough (19). The cabinet has considerable surface of phenol and water in the bottom, and the papers and the atmosphere are allowed to equilibrate with phenol and water overnight (12 to 16 hours). Seventy-five milliliters of phenol saturated with water are added to each trough through a small hole in the top of the cabinet which is closed with a stopper before and after use. Phenol solution is prepared by saturating Mallinckrodt's crystalline phenol with water and adjusting the pH to 5.0 to 5.5 with sodium hydroxide.

The phenol solution is allowed to travel downward over the paper to within 0 to 1 cm. of the lower edge. The troughs are removed from the chromatography cabinet and each paper is removed from the troughs with a special clasp, from which it is allowed to hang while being dried at room temperature in a forced draft for at least 12 hours. A walk-in drying chamber is used with a vigorous flow of air entering at the base and removed at the top.

The chromatographic procedure is repeated after folding the paper along a direction at right angles to the first fold and parallel to the long dimen-

sion of the paper. In a similar manner, collidine-lutidine is made to travel over the sheet. The collidine-lutidine is prepared by saturating a mixture of three volumes of lutidine and one volume of collidine with distilled water. The pH is adjusted to 8.5 with concentrated hydrochloric acid. Ninety ml. of collidine-lutidine solution is placed in each trough and allowed to run until all of the solvent has passed on to the paper (about 30 hours). This gives better separation and still leaves all common amino acids on the paper. Collidine-lutidine should not be allowed to run off the end of the paper until it has been determined that there are no substances in the sample which travel more rapidly than tyrosine in collidine-lutidine. The collidine-lutidine is evaporated in a forced draft at room temperatures in the same manner as the phenol.

All these operations, which involve only microgram quantities of amino acids, require scrupulous cleanliness in the handling and exposure of the papers and the actual area of the chromatogram should not be touched.

The amino compounds are made to react with ninhydrin and the density of color so produced is measured after the manner already described (**21**). When there are large quantities of amino acids, the spots will have a light area in the center; this does not have any effect on the total amount of color produced.

STANDARD CURVES.—To convert the density readings which result from the above procedure into actual amounts of amino acids, standard curves are necessary for each amino acid to be determined. The standard curves are made as follows. Two mixed standard solutions containing the amino acids are prepared. These conveniently contain 1 μg./μl. of each amino acid and the pH of the standard solution is adjusted to pH 6.5. One solution contains aspartic acid, glycine and threonine and the other contains glutamic acid, serine and alanine. The remaining amino acids may be in either solution although it is preferable that leucine be in a different solution from phenylalanine.

Different amounts of each standard solution are chromatographed and the color developed with ninhydrin (**21**). Twenty, forty, sixty, and eighty micrograms of each acid are convenient amounts and they are used in triplicate or quadruplicate. Only those values are accepted which agree within 5%. As a further check, data should only be used if they are from chromatograms on which the displaced standard does not vary by more than 5 to 7%.

The standard curves deviate, but only slightly, from linearity (see fig. 2), because the losses of amino acids are proportionately greater for small amounts of amino acids.

STANDARD CURVES FOR USE WITH UNWASHED PAPERS.—When using unwashed papers, the internal displaced standard has been utilized to measure and allow for the slight changes in the sensitivity of individual chromatograms which cannot be controlled. In compiling the standard curves, they are corrected to an accepted value for the internal standard. It has been

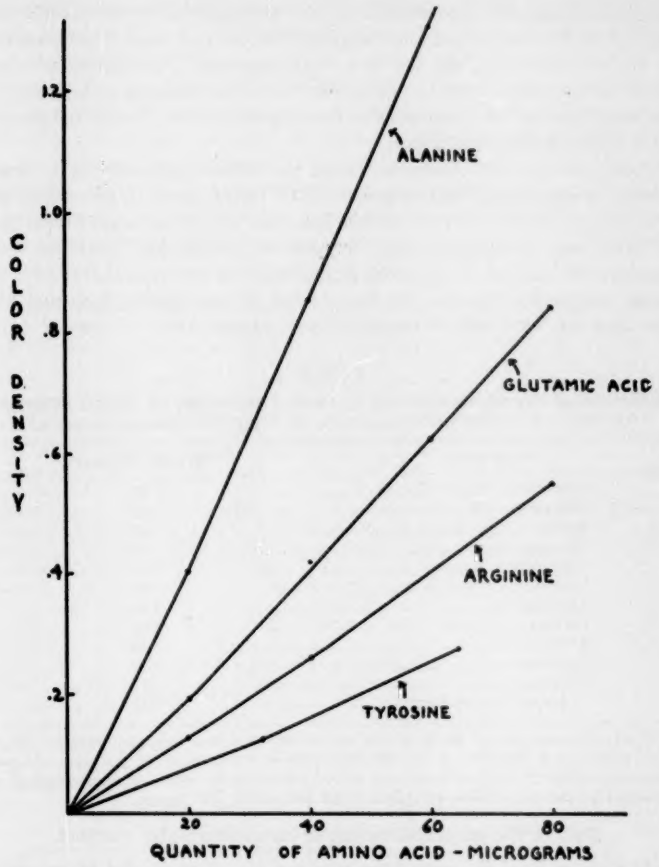

FIG. 2. Chromatography of amino acids on paper: standard curves of representative amino acids after chromatography on paper by the procedure described.

found that any variable factor which slightly increases the reading for the internal standard tends also to increase, in approximately the same proportion, the reading for all the amino acids on that paper. The calculations are made by multiplying the corrected readings (observed minus blank readings) for each amino acid, by a factor which is the ratio of the accepted value for the internal standard to the value observed for that paper. In preparing a standard curve by this means, it has been found that the effect of the correction factors so described is to bring the points even more closely to a straight line through the origin than they would otherwise be. There-

fore, in carrying out an analysis it is only necessary to obtain density readings for all the amino acids on the paper and also to make a determination on the displaced standard for that chromatogram. This procedure determines the correction factor to be applied to all the readings so that they are now calculated to the same basis as the standard curves, *i.e.*, to the accepted value of the displaced standard.

ACCURACY OF THE METHOD.—Using the above procedure, it is readily possible to determine, with an error of 10% or less, most of the amino acids in a sample. If the acid constitutes less than 1% of the total amino acids, the error may be greater. Hydroxyproline, proline and histidine should constitute at least 5% of the total amino acids for accurate analysis for with proline and hydroxyproline the blank color is unavoidably high and histidine does not react well with ninhydrin on paper.

TABLE V

CHROMATOGRAPHY OF AMINO ACIDS ON PAPER: RECOVERY OF ADDED AMINO ACIDS TO SAMPLE CONTAINING THE ALCOHOL SOLUBLE AMINO ACIDS OF POTATO.*

Amino acid	Per cent. recovery
Aspartic acid	96
Glutamic acid	100
Serine	102
Alanine	97
Glutamine	107
Lysine	99
Arginine	103
Proline	101
Valine	95
Leucine	100
Tyrosine	91
γ-Amino butyric acid	102

* Forty micrograms of each of the above amino acids (except proline—80 μg.) were added to a sample of the alcohol-soluble compounds of potato. Aliquots of a potato extract with and without added amino acids were chromatographed and measured by the procedures of this and the preceding (21) paper.

Test of the quantitative paper chromatographic method

The accuracy of the procedures outlined above was tested by measuring recovery of 40 μg. of amino acids added to a potato extract. (See table V.) In all but two cases the amount recovered is within 5% of theoretical. The fact that tyrosine gives a low recovery probably means that tryptophan and phenylalanine would also and these as well as glutamine could only be recovered with an accuracy of 10%.

Application of the method to the analysis of protein

Another comparison with other methods is the analysis of a protein that has been analyzed thoroughly before. For this purpose casein (from Merck & Company) was chosen.

The protein was hydrolyzed and analyzed as described earlier. A com-

parison of chromatographic results with recent analyses of casein is presented in table VI. The differences between the chromatographic results and those published by GORDON *et al.* (**12**) are to be seen particularly in the analysis of serine, proline and phenylalanine. This serine value was obtained consistently so that the difference may arise from the conditions of hydrolysis rather than the errors of analysis. (See corrected figures in the table.) NICOLET, SHINN, and SAIDEL (**17**) have reported that the phosphorylated serine groups in casein, vitellin, etc., undergo destruction during acid hydrolysis. The low value for threonine indicates that the β-hydroxy-amino acids may both have been destroyed during hydrolysis. The proline

TABLE VI

CHROMATOGRAPHY OF AMINO ACIDS ON PAPER: A COMPARISON OF ANALYSIS OF CASEIN BY CHROMATOGRAPHIC TECHNIQUE WITH PUBLISHED DATA.

Amino acid	Per cent. of amino acid		
	Data of Gordon *et al.* (12)	Data from paper chromatographic method	Data cited by Gordon *et al.* (12)
Cystine	.34	0.0	.34
Aspartic acid	7.1	6.9	7.2
Glutamic acid	22.4	22.6	22.0
Serine	6.3	4.8 (5.4)*	5.9
Glycine	2.7	2.3	1.9
Threonine	4.9	4.2 (4.4)*	4.6
Alanine	3.0	3.1	3.5
Lysine	8.2	8.2	8.3
Arginine	4.1	3.8	4.0
Histidine	3.1	....	3.2
Proline	11.3	12.9	11.6
Valine	7.2	6.9	7.2
Leucines	15.3	15.2	17.9
Methionine	2.8	2.7	3.1
Phenylalanine	5.0	6.8	5.5
Tryptophan	1.2	....	1.2
Tyrosine	6.3	5.7	6.2
Total	111.2	106.0	113.6

* Value corrected for losses during hydrolysis.

value may be somewhat in error because the blank correction is a relatively large part of the total reading. Phenylalanine may be in error because of the difficulty of separation from leucine. However, in general, the chromatographic analysis agrees well with the data of GORDON *et al.* (**12**). The data of Gordon were obtained using standard methods for amino acid analysis.

It can be seen from the data of table VI that analyses of protein hydrolysates by paper chromatographic methods correspond well with published analyses. The quantitative paper chromatographic method can therefore supplement those chemical and microbiological methods for amino acids already in use, because the chromatographic method is most accurate for

those amino acids which are least easily determined in other ways (*i.e.*, aspartic acid, glutamic acid, serine, glycine, threonine, alanine, valine and leucines), and it is speedy and applicable to small amounts of material.

The quantitative paper chromatographic method for determination of amino acids is therefore useful in several ways. It offers a ready and speedy means of determining most of the amino acids both in the free state and combined in the form of protein. Where greater accuracy is essential, this can be obtained by the paper chromatographic method by running several different-sized aliquots of a sample and by the use of hydrogen peroxide treatment for special purposes as already described.

Summary

1. Variables which affect the recovery of amino acids after 2-directional paper chromatography have been investigated using the quantitative ninhydrin reaction on paper as described in the previous paper.

2. Standard conditions are defined under which the chromatography of amino acids may be carried out so reproducibly so that quantitative determinations may be made using standard curves which apply once and for all to this set of conditions.

3. The method has been tested by measuring recovery of amino acids added to a plant extract. Recoveries agreed with the amounts added to within 5% or less for 10 amino acids, and to less than 10% for two others.

4. Analyses of casein have been made by the quantitative paper chromatographic method and compared with published analyses.

5. It is now evident that the method can be applied to the quantitative analysis of a wide range of amino acids whether these are present free or combined in the form of protein.

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PHYSIOLOGICAL AND BIOCHEMICAL CHANGES ACCOMPANYING POLLINATION IN ORCHID FLOWERS. I. GENERAL OBSERVATIONS AND WATER RELATIONS

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(WITH SIX FIGURES)

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Introduction

It is well known that the reproductive processes in plants are associated with pronounced changes of the physiology of the whole plant. In a series of papers, MURNEEK (**19**), MURNEEK and WITTWER (**20, 21**), WITTWER and MURNEEK (**28**) discussed mechanisms whereby synapsis and syngamy could extend their effects throughout the whole plant body. The increased knowledge about parthenocarpy has called attention to physiological distinctions between the processes of pollination and fertilization. The effect of pollination in leading to fruit development has been duplicated by use of synthetic growth substances (**10**). Fertilization is essential for embryo initiation and so far has not been replaced by treatment with any known substances. It is therefore of interest to know what effect pollination alone has on the metabolism of the flower.

In most plants under normal conditions, pollination is followed by gametic union. The fact that the two events closely follow one another makes it difficult to separate them for experimental purposes. The orchids are exceptions to the general rule; the extremely great time lag between microgametophyte and megagametophyte development in these plants has made them favorable material for "pollination effect" studies. In most orchids, pollination is necessary to start the development of the megagametophyte which is wholly undeveloped at the time of anthesis. The two processes are usually separated by a period of several months. The structure of the orchid flower offers an additional advantage for physiological investigations. The large size of the perianth and the column, a united structure composed of the stamens and the pistil, makes it suitable for studies of metabolism.

Although the "post-pollination phenomenon" has been known since Fitting's early work (**8**) on orchids, it has received relatively little further attention. The purpose of the present investigation is to find out the immediate effects of pollination, with a view to seeing to what extent these effects can be explained in terms of auxin activity. It is hoped to integrate the physiology of pollination and to derive the mechanism of its action from the results obtained.

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Literature

FITTING (**8, 9**) demonstrated that the placement of pollen, living or dead, on the stigma of orchids caused premature wilting of the perianth, closing of the stigma, swelling of the column and sometimes the swelling of the ovary. MORITA (**18**) studied the effects of application of dead pollen, foreign pollen and pollen extracts in Cymbidium. LAIBACH (**13**) reported that in Calanthe, in addition to the post-pollination phenomena shown by Fitting, dead or living pollen placed on the stigma produced curvature changes of the pedicel. That the orchid pollinium is a rich source of growth substances and therefore capable of inducing parthenocarpy and prevention of leaf abscission has been demonstrated by LAIBACH (**14, 15**), LAIBACH and MASCHMANN (**16**), and MAI (**17**). HUBERT and MATON (**11**) studied the relative effectiveness of a wide variety of chemical substances in inducing the post-floral phenomena in tropical orchids. SESHAGIRIAH (**23**) observed an abundance of starch in pollinated ovaries of Habenaria and concluded that growth substances from the pollen stimulated starch synthesis. CURTIS (**3**) reported that, in some species of Phalaenopsis, the petals do not wilt after pollination, but rather become thickened and turn green.

WHITE (**25**) studied the respiratory activity of pollinated and non-pollinated flowers of a wide variety of plants not including orchids. In almost every plant she examined, pollination not only produced a rapid rise in respiratory activity, but also markedly raised the respiratory quotient. DETURK *et al.* (**4**) noted that the beginning of phytin synthesis followed immediately after pollination, from which they concluded that pollen activated the zymogen of the enzyme responsible for phytin synthesis. The later work of EARLEY and DETURK (**5**) added some corroborative evidence supporting the above statement.

Materials and methods

In the course of this experiment more than a thousand flowers of cultivated orchids were studied. Potted plants, cut inflorescences and cut flowers, were provided through the exceptional courtesy of Dr. C. K. Schubert from his private orchid collection in Madison, Wisconsin. Some plants were raised in the greenhouses of the Botany Department of the University of Wisconsin.

Normally the flowers of a single inflorescence open at about the same time. They have approximately equal weights and are uniform in appearance. These facts made it possible to study the effects of various treatments on presumably comparable flowers from the same inflorescence.

When sufficient flowers of uniform origin were not available for an entire experiment, particular care was taken to choose like flowers for each sub-experiment, that is, each sampling, to minimize the variation not caused by treatment. In such cases, separate controls were always used for different sub-experiments.

Throughout the work, the normal complement of pollinia that is present in a particular species was used to pollinate one flower, *e.g.*, four in Cattleya and Dendrobium, eight in Laeliocattleya, etc. The pollinia usually came from flowers other than the one being pollinated, although the origin of the pollinia did not seem to make much difference.

Two growth substances were used in treating stigmas: indole-3-acetic acid and β-naphthalene acetic acid, both at a concentration of 0.5% in lanolin paste. About 5 mm.^{3} of paste was placed on the stigma of each flower treated.

The following symbols are used throughout the descriptions: Cont., Pol., NAA and IAA stand respectively for control, pollinated, naphthalene acetic acid treatment and indole acetic treatment, the letters C and P for the floral parts column and perianth. Thus, Cont-P means control perianth, NAA-C naphthalene acetic acid treated column, etc.

Almost all of the data presented are representative of many replicate sets. Unless otherwise specified, each determination involves the use of two or three large flowers such as Cymbidium or *Cattleya labiata*, and four or five small flowers such as Dendrobium or *Cattleya Bowringiana*.

Results

External changes and their quantitative analyses

In all the species studied, pollination brought about wilting of the perianth, local swelling of the column resulting in imprisonment of the pollinia, followed by enlargement of the whole column. In Cymbidium, as an immediate effect of pollination, the lip of the perianth as well as the inner or ventral side of the column turned from white to red. The color change occurred approximately 48 hours after the application of pollen and its intensity increased acropetally. The red pigment was proved to be anthocyanin. The appearance of color was not associated with any change in sap pH. All these visible effects of pollination were qualitatively duplicated by application of 0.5% NAA and IAA.

To approach the quantitative aspects of the swelling and wilting, a study was made of the relative changes in fresh and dry weights of the column and perianth. The reason for studying them separately is obvious. With pollination the life of the perianth ends; pollination sets off further growth of the column.

After treatments flowers were cut at the base of the ovary. As the ovary of most species is longitudinally grooved, the lower end of the grooves was taken as the demarkation between the ovary and the pedicel. The floral parts were weighed separately, dried at 80 to 100° C for about 24 hours and reweighed.

Pollination brought about increases in both the fresh and dry weights of the column. Since in earlier experiments a continuous growth of the control column after anthesis was indicated, later data were expressed in

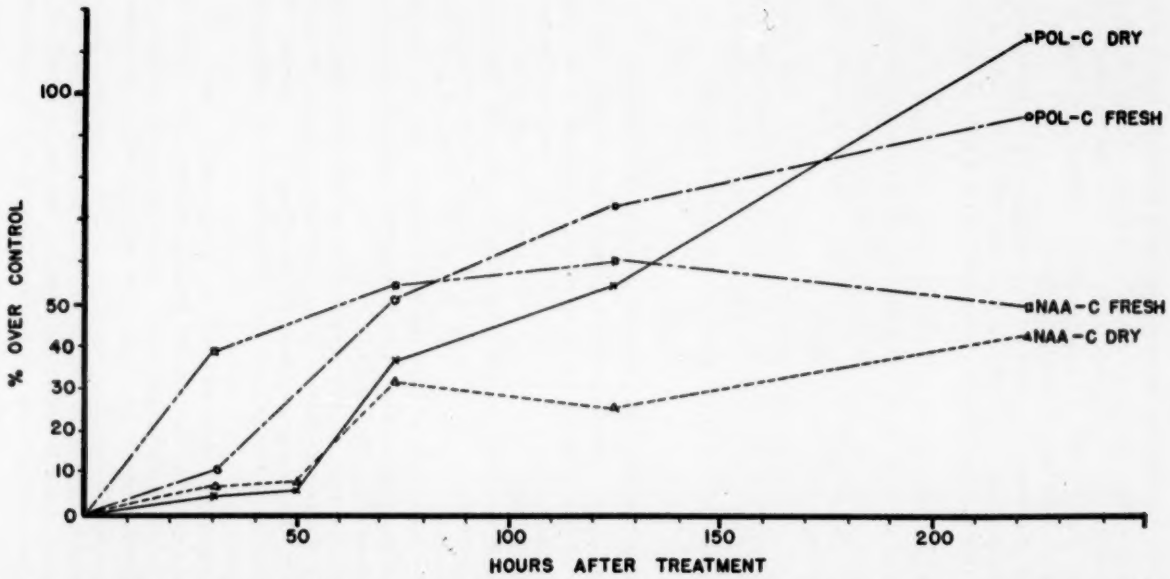

FIG. 1. Relative increase in fresh and dry weights, in per cent. over control, of columns of pollinated and NAA treated flowers. *Cymbidium Lowianum*.

per cent. of the control, so that the changes within the controls could be eliminated. Figure 1 shows the gain in fresh and dry weights of Cymbidium columns following pollination and NAA treatment. It is seen that, on a percentage basis, the increase of fresh weight was greater than that of dry weight at the beginning. Later on this relationship was reversed. These observations indicate an initial dominance of water uptake over the increase in dry matter. The same general pattern is shown in more than 20 experiments with different species. When the effect of NAA was compared with that of pollen (fig. 1), the NAA effect considerably exceeded that of pollen at the beginning, especially in increasing the fresh weight. The leveling off of the curve which soon occurred indicated that the pollen was becoming relatively more effective. The same relationship is shown in figure 2 which records the relative effectiveness of NAA, IAA, and pollen in bringing about

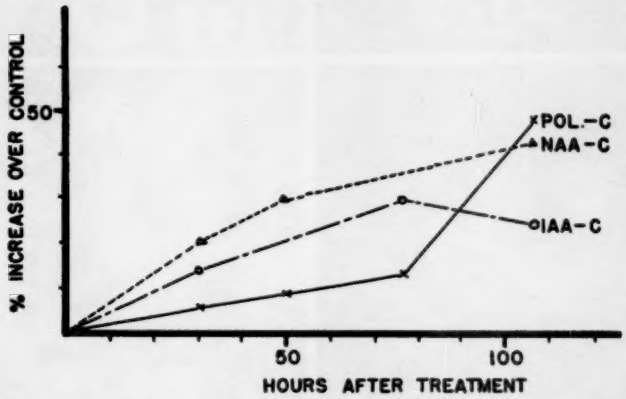

FIG. 2. Relative effectiveness of pollen, IAA, and NAA in producing dry weight increase in the column. *Cattleya labiata.*

the dry weight increase for *Cattleya labiata*. NAA had a greater effect than IAA.

The perianth not only loses water, but also dry matter. It is noteworthy that, while the fresh weight of the perianth underwent almost a linear decrease with time, the dry weight seems to be much more steady.

EXPERIMENTS ON PICKED FLOWERS

Flowers of *Dendrobium nobile* were cut from the plant and their pedicels immersed in distilled water. A wire screen fitted on a glass container was used to support the flowers. One set of these flowers was then pollinated. Figure 3 includes photographs taken 115 hours after treatments. The closing of the perianth of the flowers in the pollinated group is shown. Owing to the drying out of the tissue, the veins on the petals of the pollinated flowers are clearly visible.

The same relationship exists here between the percentage change in fresh and dry weight as revealed previously by flowers still attached to the plant, except that the magnitude of both the increase and decrease was greatly limited.

Following these preliminary observations of the apparent changes, efforts were made to determine: (1) the mechanisms governing the increased water uptake of the column and the loss of water from the perianth; (2) the relative shift of dry matter between the perianth and the column, their quantitative relationships and the components that are directly concerned; and (3) changes in the more fundamental metabolic processes such as respiration and enzymatic activity. This paper reports the findings on water relations. In a paper that will appear later the changes in respiration, catalase activity, and chemical constituents will be described.

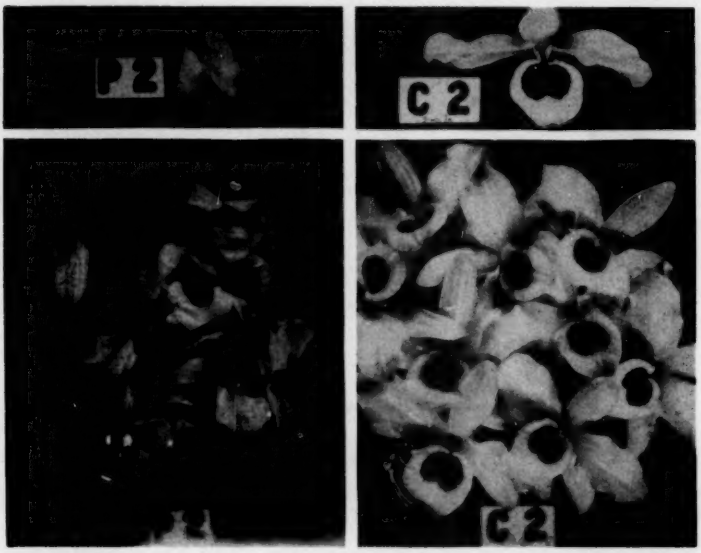

FIG. 3. Picked flowers of *Dendrobium nobile*. Left: 115 hours after pollination. Right: Non-treated, unpollinated, otherwise same as those shown at left.

MEASUREMENT OF OSMOTIC PRESSURE

Osmotic pressure was determined cryoscopically. Treated floral parts were cut from the plants and frozen with solid carbon dioxide. After thawing, the tissues were cut into small pieces and ground in a mortar before being subjected to pressing in a hydraulic press at a pressure of 8000 lb./sq. inch. Care was taken to prevent loss of water due to evaporation which would particularly affect the results with small samples. It took three

flowers of *Cattleya labiata* and eight of *C. Bowringiana* to get sufficient sap for one determination. A mixture of chipped ice and $CaCl_2$ was found satisfactory for use in the freezing bath. Freezing point determinations were made with a Drucker-Burian microthermometer.

The results are summarized in table I. A slight increase of osmotic pressure in the column was shown 115 hours after pollination and auxin treatment. The change in osmotic pressure of the perianth followed the same pattern.

Uptake of distilled water by cut discs

Experiments were carried out to compare the ability of detached flowers to take up water. For this purpose, the flowers were severed from the plants at various intervals after treatment and separated into column and perianth. The respective parts were hand-sectioned, the weights of the sections obtained and the sections put into bottles containing enough distilled water to cover them. The sections turned brown very quickly in contact with air, the pollinated ones at a greater rate and intensity. No attempts were made to prevent oxidation, since any such effort might result in an interference with water uptake. The sections were taken from the water after two to three hours and reweighed after the adhering water had been blotted off with filter paper. The gains in weight are recorded as a percentage of the original weight in the left-hand portion of table II. It is obvious that the column of the pollinated flower took up significantly more water from the medium.

TABLE I

EFFECTS OF POLLINATION AND AUXIN TREATMENT ON OSMOTIC PRESSURE OF FLORAL PARTS. DETERMINATIONS WERE MADE 115 HOURS AFTER TREATMENT.

Part and treatment	Osmotic pressure in atmospheres			
	Cattleya labiata		*C. Bowringiana*	
	Expt. 23	Expt. 25	Expt. 23	Expt. 25
Cont-C	5.99	5.76	5.91	4.43
Pol-C	6.80	6.34	6.37	....
NAA-C	....	....	....	5.16
Cont-P	5.22	5.93	4.33	5.40
Pol-P	6.49	....	5.37	....
NAA-P	....	....	....	6.29
IAA-P	6.82	....	....	....

It was suspected that equilibrium might not have been reached at the time determinations were made, and that the observed differences in the amount of water uptake might disappear when more time was allowed. However, a study of the rate of water uptake with Laeliocattleya showed that the treated and non-treated tissues reached their saturation point at about the same time. Figures 4 and 5 show that the percentage difference

TABLE II

UPTAKE OF DISTILLED WATER, IN PER CENT. OF ORIGINAL WEIGHT, BY CUT DISCS FOLLOWING VARIOUS TREATMENTS, IN AIR AND IN NITROGEN.

Part and treatment	In air					In nitrogen		
	Expt. 37 *Cattleya labiata*		Expt. 45 *Cattleya labiata*		Expt. 68 *Dendrobium nobile*	Expt. 47 *Cattleya labiata*	Expt. 48 *Cattleya labiata*	Expt. 73 *Cattleya hybrid*
	17 hrs.	45 hrs.	25 hrs.	54 hrs.	48 hrs.	74 hrs.	25 hrs.	48 hrs.
Cont-C	3.42	6.90	8.80	6.60	3.64	8.35	8.11	2.67
Pol-C	6.46	9.40	10.76	9.12	11.70	7.74	8.11	2.23
NAA-C	10.06	12.71	....	....	10.40	....	5.20	....
IAA-C	7.95	9.87	....	....	....	....	....	....
Cont-P	16.26	10.65	16.50	14.78	14.22	8.45	12.20	....
Pol-P	19.02	15.36	17.98	16.78	29.51	12.49	20.42	....
NAA-P	21.94	14.31	....	....	39.36	....	....	....
IAA-P	19.85	19.81	....	....	....	....	....	....

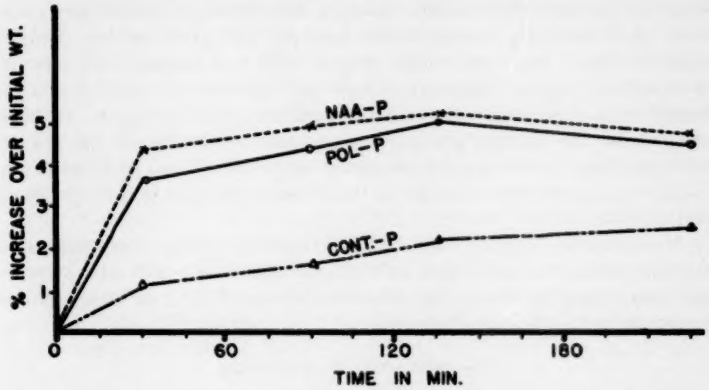

FIG. 4. Rate of water uptake of detached perianth from differently treated flowers. Each point on each curve represents increase in weight in percentage of initial weight. Laeliocattleya.

was established significantly within 30 minutes after the tissues were immersed in water.

To determine whether this accelerated water uptake was in any way connected with aerobic processes, experiments were run in an atmosphere of nitrogen. Commercial nitrogen was purified by passing through the following solutions: sodium hydrosulphite (with indicator, sodium anthraquinone β sulphonate, added) to absorb oxygen; NaOH to absorb carbon dioxide. This system was finally connected to a water trap to regulate the rate of gas

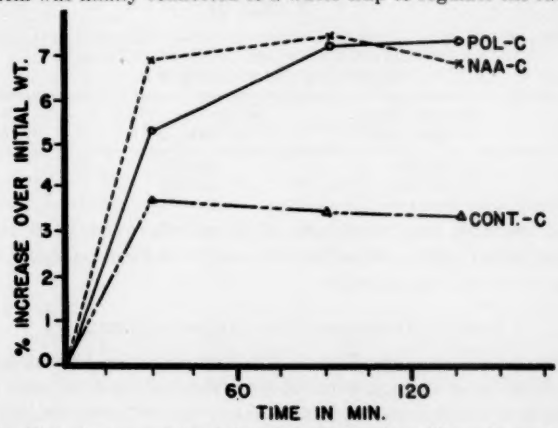

FIG. 5. Rate of water uptake of detached columns from differently treated flowers. Each point on each curve represents increase in weight in percentage of initial weight. Laeliocattleya.

flow. In the earlier experiments, nitrogen was passed to a closed jar. Sections from differently treated flowers were put into small bottles. Only a small amount of water was added so as to hold at a minimum the amount of dissolved oxygen. The bottles were put into the jar which had been flushed with a nitrogen stream for a sufficient period of time. In later experiments, the nitrogen gas was passed continuously through the bottles at a moderate rate during the experimental periods of two to three hours. The percentage increase in weight of the tissues is recorded in the right-hand portion of table II.

It is clear that in an atmosphere of nitrogen the columns from pollinated and non-pollinated flowers took up the same amount of water on a percentage basis. In other words, the effects of pollen and NAA on water uptake disappear in the absence of oxygen.

Possible role of imbibition

Ovaries of pollinated flowers of many species of orchids seem to contain more mucilaginous substances than those of non-pollinated flowers. This fact was especially noticeable during the process of sectioning and grinding the experimental material. This observation led to the speculation that imbibition by such substances might explain part of the increase in water uptake.

To investigate this possibility, water uptake by killed tissues was studied. Fresh columns were weighed and killed by steaming, after which they were soaked in distilled water. As can be seen in table III, at the end of three hours, both the pollinated and the non-pollinated tissues lost some weight to the medium, but the latter to a much less extent than the former.

TABLE III

WATER LOSS FROM COLUMNS OF *Dendrobium nobile* FROM POLLINATED AND NON-POLLINATED FLOWERS. KILLED BY STEAMING ONE WEEK AFTER POLLINATION.

Tissue	Original fresh weight, gms.	Weight after 3 hrs. in water, gms.	% decrease in weight
Cont-C	0.5285	0.3877	26.64
Pol-C	0.7575	0.6466	14.64

While this is not conclusive proof of the role played by imbibition in the accelerated water uptake, it indicates that after pollination the column has a greater water-holding capacity.

Loss of water from perianth—transpiration

The transpiration of the flowers was studied in the following manner. Flowers of like size and age were cut from the plant, treated and weighed. The pedicels of the flowers were immediately inserted into the rubber caps of small flasks or vials filled with distilled water. The holes in the rubber caps were made to fit the pedicels. To ensure complete sealing, the flower-

to-rubber and rubber-to-glass connections were sealed with a mixture of paraffin and beeswax. The flasks were weighed at intervals of about 12 hours. The decrease in weight is taken as the loss of water through the flower, presumably mainly the perianth. Figure 6 plots the loss of water per unit time per unit weight of the flower against time. The choice of unit weight here is thought to be reasonable because the petals are flat and can thus be taken as a 2-dimensional structure and any change in surface would be reflected in the weight.

It is readily seen that both pollination and auxin treatment evenly and continuously stimulated transpiration from the beginning to the end of the experiment. Eight different experiments showed the same general result.

Microscopic examinations revealed no stomata in the epidermis of the perianth. Therefore it must have been the epidermal or cuticular transpiration which was affected.

Discussion

Evidence has been presented to show that the placement of pollen on the stigma greatly shifts the water balance of the flower. The column swells mainly due to increased capacity to absorb water; the perianth wilts mainly because of increased transpiration.

The application of NAA and IAA in place of pollen to the stigma produces qualitatively the same results. We shall discuss the mechanism of pollination, together with the comparison of the pollen effects and auxin effects, in the second paper, and confine our discussion here to the effects of pollen or auxin on water uptake and transpiration.

The effect of auxin on water uptake has been summarized in review papers by VAN OVERBEEK (**27**) and SKOOG (**24**). In the present work, auxin did not cause a decrease of osmotic pressure as reported with potato tubers by VAN OVERBEEK (**26**). However, the same conclusion is reached that the slightly increased osmotic pressure is far from being enough to explain the enhanced water uptake. This increased water uptake is made evident in the apparent swelling of the column and definitely proved by the experiments on cut discs of column tissue in water.

It is shown that cut discs from columns of treated flowers absorb more water than those from columns of non-treated flowers in air, this difference disappears in a nitrogen atmosphere. This dependence of pollen- and auxin-stimulated water uptake on oxygen is in agreement with the results obtained by KELLY (**12**) in Avena coleoptile segments.

As to the effect of auxin on transpiration of plants, there seems to be a lack of agreement among the earlier authors. AMLONG (**1**) reported an increased transpiration in tomato plants from seeds that had been soaked in a solution containing potassium naphthalene acetate. BROWN (**2**) presented data showing the amount of water transpired by normal bean seedlings and by those sprayed with 2,4-D and concluded that the treated seedlings transpired significantly less water than the non-treated ones. The results of FERRI and LEX (**6**), FERRI and RACHID (**7**) are also in favor of

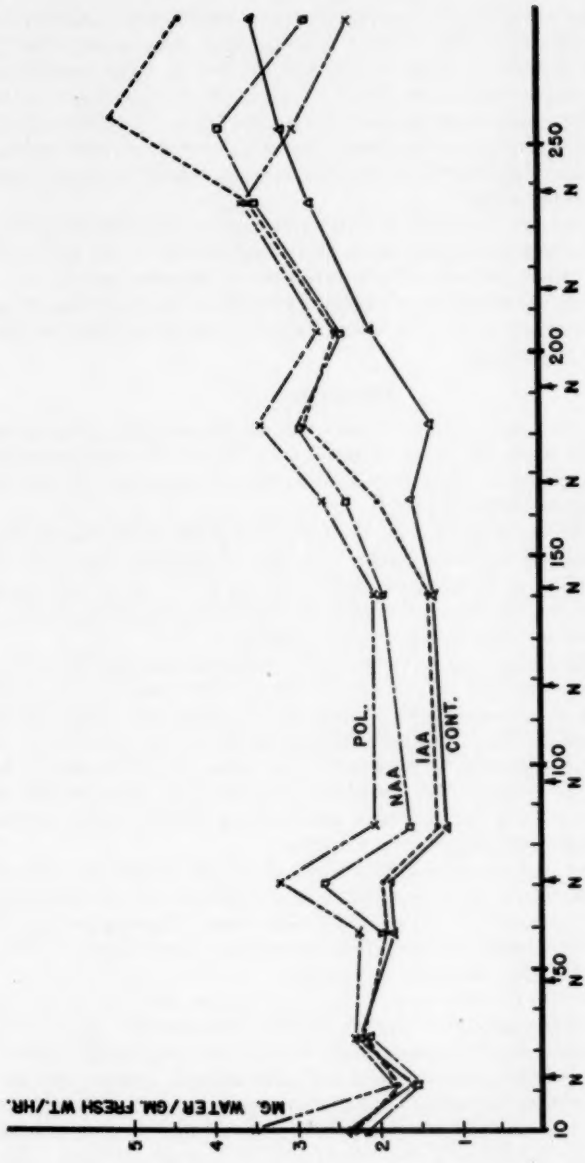

FIG. 6. Rate of transpiration of flowers following various treatments. *Cattleya labiata*.

the conclusion that treatment of plants with growth substances generally reduces water loss from the plants. Lately, PLAYER (**22**) noticed a decrease of total transpiration in castor bean plants sprayed with various synthetic growth substances, although the total transpiration of corn plants was not affected by these sprays.

In the present investigation, the method of application of auxin is different from all the earlier ones, and the plant parts concerned are also different. Auxin is applied to the stigma and the transpiration of the perianth is studied. This may not be comparable to spraying the plants and studying the transpiration of the leaves. However, our conclusion that auxin treatment increases transpiration rate would not conflict with BROWN'S (**2**) conclusion if he had taken leaf surface into consideration. He shows in his table 1 that the loss of water per 10 plants in five days is 75.41 and 49.88 grams respectively for the untreated and treated seedlings. But during this period, the untreated plants have grown to be twice as large with a leaf surface twice as great (see his table 4). Recalculation of his data with consideration of the leaf surface gives a relative transpiration rate of roughly 37.5 to 50 for the untreated and the treated. An acceleration of transpiration rate is therefore caused by the treatment with 2,4-D.

Summary

Pollination was shown to bring about the external visible changes reported by earlier workers as to the wilting of perianth, the closing of the stigma, the enlargement of the column, and sometimes the swelling of the ovary. In Cymbidium, as an immediate effect of pollination, the lip of the perianth as well as the inner side of the column turned from white to red. The red pigment was proved to be anthocyanin. The appearance of color was not associated with any change in sap pH.

Pollination brought about increases in both fresh and dry weights of the column. On a percentage basis, the increase in fresh weight was greater than that of dry weight at the beginning; later on this relationship was reversed. The greater per cent. increase of fresh weight over dry weight, followed by a reversion of this relationship, indicates an initial dominance of water uptake over the increase in dry matter.

The perianth after pollination not only lost water, but also dry matter. Cut flowers responded to pollination in essentially the same manner.

Osmotic pressure measured 115 hours after pollination showed a slight increase over the control. This slight observed difference in osmotic pressure did not appear to be sufficient to account for the difference in the amount of water uptake.

In air, cut discs of columns from pollinated flowers took up more water on a percentage basis than those discs of columns from non-pollinated flowers. This difference was significantly established within 30 minutes after the tissues were immersed in water. However, in a nitrogen atmosphere, this difference disappeared. It is concluded that the pollen- or auxin-stimulation of water uptake is related to aerobic processes.

Columns from pollinated flowers were shown to have a greater water-holding capacity. It is postulated that pollination causes an increase of hydrophilic colloids in the column tissue.

The wilting of the perianth after pollination and auxin treatment results from an increased rate of epidermal transpiration.

The author wishes to express her gratitude and appreciation to Dr. C. K. Schubert of Madison, Wisconsin, without whose exceptional kindness in contributing the flowers from his private orchid collection the investigation would have been impossible; to Dr. John T. Curtis for suggesting the problem and for his interest and helpful guidance throughout the course of the study; to Dr. J. F. Stauffer for his help in various ways; to Dr. Hilda F. Rosene for her constructive criticism on the part of water relations; to Dr. W. Gordon Whaley for reading the manuscript. She is also indebted for the fellowships awarded during her studies at the University of Wisconsin.

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GROWTH OF *ANANAS COMOSUS* (L.) MERR., AT DIFFERENT LEVELS OF MINERAL NUTRITION UNDER GREENHOUSE AND FIELD CONDITIONS. II. CHEMICAL COMPOSITION OF THE TISSUES AT DIFFERENT GROWTH INTERVALS[1]

C. P. Sideris and H. Y. Young

(WITH SIX FIGURES)

Received October 2, 1950

This report supplements the earlier report (**13**) which discussed the growth rates, water and mineral nutrient intake by the same group of plants. The data herein considered deal with the chemical composition of the leaf and stem tissues at different growth intervals of pineapple plants grown in nutrient solutions in the greenhouse and in soil under field conditions with different amounts of nitrate, potassium, and calcium.

The plant tissues were analyzed for total nitrogen, soluble organic nitrogen, potassium, calcium, reducing and total sugars; the latter hydrolyzed by invertase. The methods for potassium and calcium were reported in a previous publication (**9**). Total nitrogen and soluble organic nitrogen were determined by the Kjeldahl procedure, using selenium oxychloride as catalyst in the oxidation of organic matter. The fractionation of total and soluble organic nitrogen was conducted according to technique reported previously (**7, 8**). Reducing sugars, total sugars, and sucrose, the latter by the difference between total and reducing sugars after hydrolysis with invertase, were determined according to the method of Quisumbing and Thomas (**6**). Starch was determined by the method of Pucher and Vickery (**5**).

Results

Greenhouse plants

The chemical composition of the nutrient solutions in the different cultures, reported previously (**13**), is briefly repeated for the sake of convenience, as follows: concentrations in micrograms per milliliter of NO_3 and Ca for cultures A, B, C, D, and E were, respectively, 14 and 20, 28 and 40, 56 and 80, 112 and 160, and 140 and 195, with K constant at 78 γ. Similarly, concentrations of NO_3 and K for cultures F, G, H, I, and J were, respectively, 14 and 19, 28 and 39, 56 and 78, 112 and 156, and 140 and 234, with Ca constant at 80 γ. All other nutrients were maintained at approximately constant concentrations in all cultures, as much as possible.

The data on nitrogen, calcium, potassium, sugars, and starch in the basal, no. 1, and in the chlorophyllous, no. 4, sections of the active D leaves (**8**)

[1] Published with the approval of the Director as Technical Paper no. 200 of the Pineapple Research Institute of Hawaii, Honolulu, Hawaii.

for the growth periods of January, April, and July 1943, in the various figures and table I, reveal information as follows:

Nitrogen

Results, in figure 1, show that total nitrogen in the non-chlorophyllous basal sections of the active D leaves, of meristematic status until the period of floral differentiation, decreased with advancing physiological and chronological age in all cultures except in I and J. The changes in the physiological activities of the tissues of the basal sections of the active D leaves, resulting from the conversion of their status from meristematic to specialized with advancing age, increased lignification and decreased the water

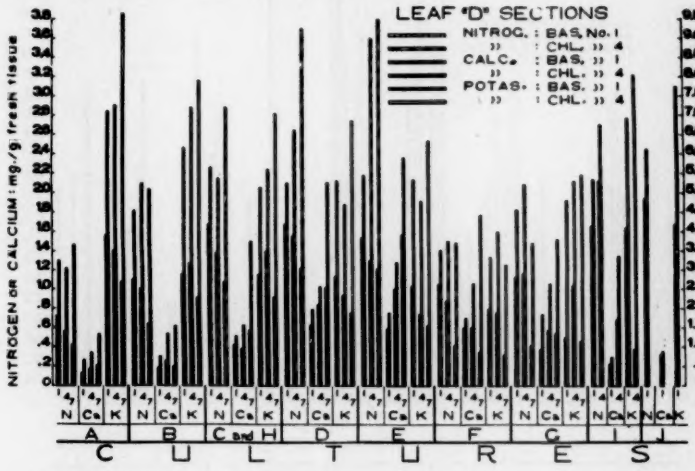
LEAF "D" SECTIONS
NITROG. : BAS. No. 1
» : CHL. » 4
CALC. : BAS. » 1
» : CHL. » 4
POTAS. : BAS. » 1
» : CHL. » 4
NITROGEN OR CALCIUM: mg./g. fresh tissue
POTASSIUM: mg./g. fresh tissue
A B C and H D E F G I J
C U L T U R E S

Fig. 1. Total nitrogen (N), calcium (Ca), and potassium (K) in the non-chlorophyllous basal, no. 1, and chlorophyllous, no. 4, leaf sections of the active D leaves in January (1), April (4), and July (7) for cultures A, B, C or H, D, E, F, G, I, and J.

content, the former by lowering the hydrophilism of the colloidal suspended matter in the cells and nitrogen concentrations per unit of tissue weight, and the latter by dilution with greater depositions of inorganic and organic matter reserves.

In the chlorophyllous distal, no. 4, sections of the active D leaves, total nitrogen remained almost at constant levels in the low-NO_3 cultures A, B, F, and G, but increased in the high-NO_3 cultures C, D, E, H, I, and J with advancing physiological and chronological age. The differences in the total nitrogen content of the tissues between the low-NO_3 and high-NO_3 cultures may be attributed to differences in the rates of intake of NO_3 from the nutrient solutions and formation of tissue matter; in the low-NO_3 cultures the rates of formation of tissue matter exceeding those of NO_3 intake and

the reverse in the high-NO_3 cultures. The physical or chemical composition of the tissues in the chlorophyllous distal, no. 4, sections of the active leaves did not undergo as great changes, between the prefloral and postfloral stages, as the basal sections.

Total nitrogen concentrations of the stem for comparable growth intervals, in figure 2, correlated positively with the nitrate concentrations in the nutrient solutions of the various cultures. However, similar concentrations in the stem between early and late growth intervals indicated decreases, resulting from advancing physiological and chronological age. For example,

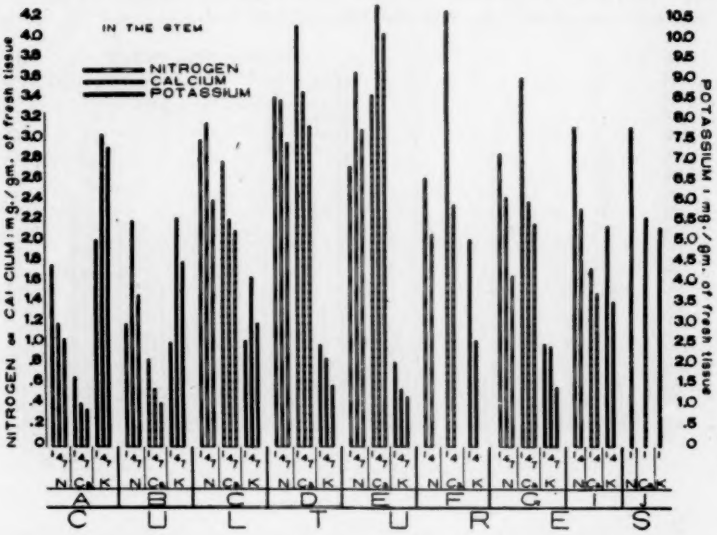

FIG. 2. Total nitrogen (N), calcium (Ca), and potassium (K) in the apical sections of the stem in January (1), April (4), and July (7) for cultures A, B, C or H, E, D, F, G, I, and J.

total nitrogen concentrations in the stem for cultures A, D, F, G, and I decreased with advancing plant age, while for B and E they fluctuated, and for culture J, with a single determination, the outcome of the results was entirely indefinite.

Soluble organic nitrogen concentrations in the non-chlorophyllous basal, no. 1, and in the chlorophyllous distal, no. 4, sections of the leaves, and in the medial and apical sections of the stem, in figure 3, correlated positively with the concentrations of nitrate in the nutrient solutions of the various cultures. However, in the stem, soluble organic nitrogen concentrations fluctuated slightly, decreasing in the low-N cultures or increasing in the

high-N cultures with advancing plant age, presumably due to differential rates of utilization in the formation of new tissues.

Soluble organic nitrogen concentrations in the leaves of the low-nitrate culture A decreased due to insufficient intake of NO_3, whereas in cultures B, C, and G, with intermediate nitrate, it remained approximately constant, but increased in the high-nitrate cultures D, E, I, and J, presumably due to greater rates of NO_3 intake and assimilation than of utilization as soluble organic-N in protein synthesis for the formation of new tissues.

Calcium

The concentrations of this element in the non-chlorophyllous basal, no. 1, and in the chlorophyllous distal, no. 4, sections of the active D leaves, in

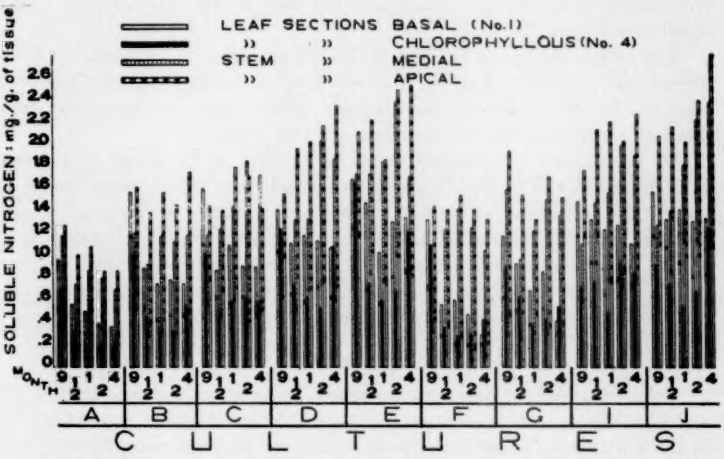

Fig. 3. Soluble organic nitrogen in the non-chlorophyllous basal, no. 1, and chlorophyllous, no. 4, sections of the leaves and in the medial and apical stem sections, in September (9), December (12), January (1), February (2), and April (4) for cultures A, B, C or H, D, E, F, G, I, and J.

figure 1, increased with advancing chronological age from January to April and July, except in the basal sections of the F culture. It is possible that the low-nitrate concentrations in the F culture might have effected a low uptake of calcium. In culture A, with lower calcium than in F but with equal nitrate in the nutrient solution, calcium uptake was considerably smaller in the early stages of growth, but increased with advancing plant age, whereas in culture F it decreased with such age. Comparable fluctuations in Ca concentrations were not observed for the chlorophyllous, no. 4, sections where Ca increased with advancing chronological and physiological plant age, in all cultures.

In cultures A, B, C, D, and E, calcium intake correlated positively with the concentrations of this element in the nutrient solutions. However, in cultures F, G, H, I, and J, with equal concentrations of calcium in the nutrient solutions, calcium intake correlated negatively with the concentrations of potassium in the same solutions, suggesting antagonistic effects.

Calcium concentrations, in figure 2, were considerably greater in the stem than in the leaves for the same cultures. Previous studies (9) indicated that calcium accumulated more at the meristematic apical regions of the stem in plants of prefruiting than postfruiting status. However, with the formation of the peduncle and the fruit and the termination of the apical stem meristematic tissues during the fruiting stage, the high calcium accumulations in the stem of the prefruiting stage decrease considerably, reappearing gradually in the tissues of the stem of the crown, or top, at the upper terminus of the fruit.

The accumulations of calcium in the apical regions of the stem of plants of prefruiting stage may result from low rates of calcium mobility from the stem to the leaves through meristematic tissues with incomplete development of conducting vessels connecting the two organs.

Of interest are the concentrations of calcium in the stems of cultures D or E and F, the former two with twice and three times, respectively, as high concentrations of calcium in the nutrient solutions as the latter, showing that culture F contained as high calcium in the stem in January as E and D. The high calcium content of the F culture, presumably, resulted from a lower intake of, and lack of antagonism by potassium, than in cultures D or E, with greater concentrations of potassium.

Potassium

Potassium concentrations in the active D leaves of cultures A, B, C, D, and E, in figure 1, with constant amounts of K but different amounts of Ca in the nutrient solutions, correlated negatively with the amounts of Ca in the solutions. In cultures F, G, H, I, and J, with constant concentrations of Ca but variable K in the nutrient solutions, potassium in the active D leaves correlated positively with the concentrations of this element in the nutrient solutions.

In the non-chlorophyllous basal, no. 1, sections of the leaves, potassium decreased with advancing plant age, although exceptions may be noted in cultures B, C, and G. In the chlorophyllous distal, no. 4, sections, potassium increased with advancing plant age, except in culture F, with very low K in the nutrient solutions.

Potassium concentrations in the stem, in figure 2, decreased in cultures A, B, C, D, and E in inverse proportion to the calcium concentrations in the nutrient solutions. In cultures F, G, I, and J, potassium concentrations of the stem fluctuated, thus precluding logical interpretation of the results, especially with a smaller number of determinations than in A, B, C, D, and E.

The chlorophyllous distal, no. 4, leaf sections contained from one to four times as much nitrogen, calcium and potassium as the basal non-chlorophyllous sections. The greater accumulations of nitrogen in the chlorophyllous than non-chlorophyllous sections were related to the greater concentrations of chloroplastic proteins in the former. However, potassium and calcium accumulated more in the chlorophyllous sections as a result of the acropetal movement of the transpiration stream in Ananas and other monocotyledonous plants.

Sugars

Previous results (**8, 10, 12**) revealed that sugar concentrations in the leaves were intimately associated with the metabolic activities of the tissues of different sections. For example, sugar concentrations in chlorophyllous distal sections were generally related to the rates of photosynthesis and of translocation to other parts of the plant, while in non-chlorophyllous basal sections they were related to the rates of translocation from the chlorophyllous sections and of utilizations by meristematic tissues in growth or of polymerization in specialized storage tissues.

In the sections of the stem and active D leaves, sugar concentrations varied from September 1942 to July 1943 for the different cultures, as depicted in figures 4, 5 A and 5 B. For instance, sugar concentrations in the stem, peduncle and fruit, in figure 4, fluctuated considerably in all cultures at various growth intervals. Cultures A, B, C, D, and E were without definite trends of sugar accumulations either in the high- or low-nitrate cultures, while in F, G, H, I, and J, greater sugar accumulations in the stems were associated with the low-nitrate cultures. In the fruits, sugar concen-

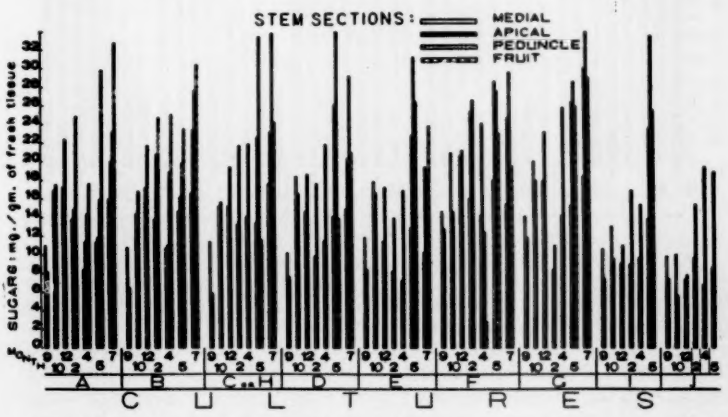

FIG. 4. Total sugars in the medial and apical sections of the stem and in the peduncle and fruit, in September (9), October (10), December (12), February (2), April (4), May (5), and July (7) for cultures A, B, C or H, D, E, F, G, I, and J.

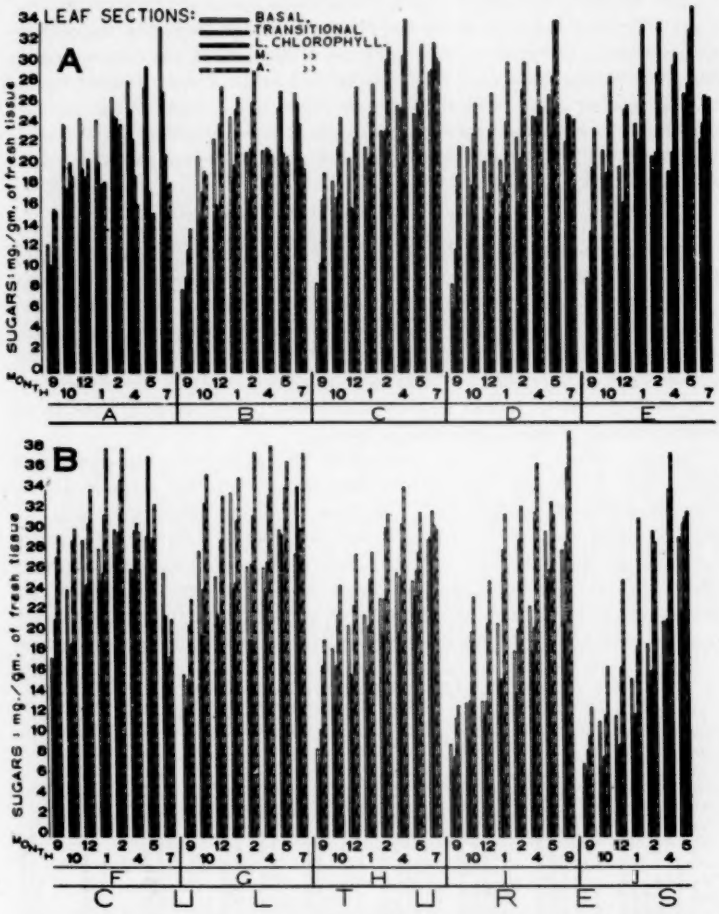

FIG. 5 (A & B). Total sugars in the non-chlorophyllous basal, transitional, and chlorophyllous (L. (low), M. (medial) and A. (apical)) leaf sections, in September (9), October (10), December (12), January (1), February (2), April (4), May (5), and July (7) for cultures A, B, C, D, E, and F, G, H, I, J.

trations were greater in cultures A, B, and C, with low nitrate, than in D and E, with high nitrate, presumably due more to the advanced chronological age and lower rates of growth of the fruits of the former. Also, the fruits of cultures A, B, and C, resulting from earlier periods of floral differentiation, were at more advanced stages of development and required less sugars for the formation of new tissues than the fruits of cultures D and E.

Moreover, the final mean fruit weights for cultures A, B, C, D, and E were, respectively, 1500, 1600, 1700, 1850, and 2100 grams, suggesting differential rates of sugar utilization for the different cultures.

Sugar fluctuations in the stems of the different cultures at various growth intervals were presumably related to different rates of production by photosynthesis in the leaves, translocation to and utilization by the meristematic tissues, for new growth, in the stem, leaves or fruit.

Sugar concentrations in the leaves, in figures 5 A and 5 B, showed certain differences between the non-chlorophyllous tissues of meristematic status of the basal sections and those of the chlorophyllous-transitional, -low, -medial, and -terminal sections (**8**), which resulted from differences in physiologic function.

For instance, malic and citric acid concentrations, at night or under reduced light conditions, were greater in the chlorophyllous than non-chlorophyllous tissues of the same leaves (**10, 11, 12**), due to differences in the metabolic activities of same. Also, sugar concentrations, in inverse relation to organic acids, varied between chlorophyllous and non-chlorophyllous tissues of the leaves. However, differences in sugar or organic acid concentrations between comparable tissues of leaves of different plants were related to differences in the rates of growth of said plants (**12**).

Sugar concentrations for culture A, in figure 5 A, were higher in the basal than in all other sections of the active D leaves at all growth intervals, except in September 1942 and May 1943, in the early and late stages of growth. The association of high sugar concentrations with the tissues of the non-chlorophyllous basal sections of meristematic status suggests that the rate of nitrate intake by the roots from the external solution was much lower than that of translocated sugars from the chlorophyllous sections for utilization in new growth. Sugar concentrations in culture F, in figure 5 B, with low potassium but with nitrate concentrations equal to A, were considerably lower in the non-chlorophyllous basal than in the chlorophyllous distal sections, suggesting conditions other than high rates of utilization for the low sugar values. Sugar concentrations in the chlorophyllous distal sections of culture A was approximately 170% as high as in F. This difference might suggest much lower rates of sugar translocation from the chlorophyllous distal to the non-chlorophyllous basal sections in culture F.

Previous studies (**10**) indicated that sugar accumulations in the chlorophyllous distal sections of leaves were greater in low- than high-potassium cultures, a condition possibly attributable to lower rates of translocation from the chlorophyllous to the non-chlorophyllous basal sections. Also, sugar polymerization by conversion to starch or other polysaccharides, being greater in high-potassium cultures (**10**), might account for the greater sugar accumulations in culture F, with low-potassium, than in A, with high-potassium. Moreover, culture F, with smaller plant weights than A, might suggest lower rates of sugar utilization with consequent accumulations in all leaf sections.

During the growth intervals of May and July, following the cessation of new growth in the leaves and stems except in the fruits, and decreased sugar consumption, sugar concentrations were much greater in the basal than in the chlorophyllous distal sections for cultures A and F. There is, also, the possibility that decreased photosynthetic activity due to aging might account for the lower sugar concentrations in the chlorophyllous than in the non-chlorophyllous sections for May and July than for previous intervals. Previous experiments (**10**), reporting differences in the sugar and organic acid concentrations in the tissues of new and old leaves, have indicated that aging, affecting the photosynthetic activity, caused reductions in the concentrations of both substances.

Sugar concentrations were greater in the chlorophyllous than the non-chlorophyllous sections of the active D leaves of culture B than of A. The results suggest that in culture B, with twice as great supplies of nitrogen as A, the rate of sugar utilization in the meristematic tissues of the basal sections was greater. It is also possible that in culture B, with greater leaf growth than A, the rate of sugar production by the chlorophyllous distal sections presumably exceeded that of utilization by the non-chlorophyllous basal sections, consequently resulting in greater accumulations.

Sugar concentrations in culture G, in figure 5 B, differed only slightly from those in F, except in May and July, when they were greater in the chlorophyllous sections of G. Such differences should be attributed to the greater nitrate intake of the G cultures which, supplying more nitrogen for the requirements of the chloroplastic proteins, presumably increased the photosynthetic efficacy of the chloroplastic tissues.

In cultures C or H, sugar concentrations were greater in the chlorophyllous distal than in the non-chlorophyllous basal or transitional sections at all growth intervals. Contrasting culture C with A or B, sugar concentrations in the chlorophyllous distal sections were greater in the former than latter cultures, due to greater nitrate intake, and its consequent favorable effects on chloroplastic proteins. However, in the non-chlorophyllous basal sections, sugar concentrations at most growth intervals were greater for cultures A and B than for C, resulting from lower rates of sugar utilization for the former.

Sugar concentrations in both non-chlorophyllous and chlorophyllous sections were lower in culture H, identical to C, in figure 5 B, with higher external supplies of potassium than in cultures F and G, presumably due to sugar polymerization by the higher potassium concentrations in the tissues.

Sugar concentrations in cultures D and E, with twofold and fourfold as great nitrate concentrations, respectively, as C, differed very little from the latter, presumably due to small differences in nitrate intake and rates of assimilation between the former and latter cultures (**13**).

The nitrate content of cultures C or H, *i.e.*, approximately 0.9 gm. in 17 liters of nutrient solution, as indicated by the various data, was at the optimal requirements of the plants for the six weeks' intervals. Chemical

analyses of these nutrient solutions at the end of six weeks, the time the old solutions were replaced by new ones, showed residual nitrate concentrations approximately 10 γ per milliliter, while those of A, B, F, or G were completely exhausted. Higher NO_3 concentrations in cultures D, E, I, and J than in C or H caused, in the former, slightly greater rates of plant growth and nitrate absorption and sugar utilization in the non-chlorophyllous basal leaf sections than in the latter, but at no time complete exhaustion of nitrate in the nutrient solutions.

In culture I, with considerably greater stem growth and total starch reserves, sugar concentrations were smaller than in D, presumably due to continuation of vegetativeness in the former and utilization of sugars for growth, except in one of 16 plants which fruited.

From the point of view of general observation, the plants of the different cultures varied in stem, leaf, and root weights (**13**). Also, the leaves had different degrees of greenness, or amounts of mottling or necrosis at the terminal regions. In the low-nitrate cultures A and F, the mature C and active D leaves developed, following the period of flowering, mottling of yellowish to brown areas at the terminal regions which gradually extended downward to approximately one fourth or more the length of the leaves. Such areas gradually became chlorotic, and in severe cases necrotic or dried, reducing considerably the photosynthetic area and efficacy of the leaves. Similar conditions were not observed in the high-nitrate cultures, indicating that nitrogen insufficiency in the low-nitrate cultures during the period of rapid fruit growth was responsible for the mottling and necrotic condition of the terminal tissues of the leaves. Unpublished data have indicated that terminal leaf necrosis during the fruiting stage is associated with the withdrawal of water and food reserves from mature and old leaves for the promotion of fruit or new vegetative growth.

Field grown plants

Results from plants grown in the field from October 1943 to August 1945, with 500 pounds of nitrogen, as ammonium sulphate, and 0, 200, 400, 800, 1600 and 3200 pounds of K_2O, as potassium sulphate, in solution, are given in table I; also, mean fruit and slip weights of same were reported in figure 5 C of a previous publication (**13**).

The data in table I, showing mean weights of stems and leaves per plant for the various treatments and total or absolute amounts of certain substances in these organs, the latter calculated from the products of the concentrations of the various substances, in figure 6, by the weights of the stem or leaves, in table I, indicate, for the greater part, positive correlation between said substances and the weights of the stems or leaves. For instance, in table I, absolute amounts of dry matter, total sugars, sucrose, total nitrogen, protein-N and soluble nitrogen correlated positively with stem weights, with coefficients, r, of 0.96, 0.91, 0.92, 0.99, 0.99, and 0.87, respectively, statistically significant at P = .01. Correlations between abso-

TABLE I

MEAN TOTAL WEIGHTS OF STEMS AND LEAVES PER PLANT AND RATIOS OF SAME FOR TREATMENT X TO X, A, B, C, D AND E; ALSO, TOTAL AMOUNTS, PER PLANT STEM OR LEAVES, OF DRY MATTER, TOTAL- AND REDUCING-SUGARS, SUCROSE, STARCH, TOTAL-, PROTEIN- AND SOLUBLE ORGANIC-NITROGEN, IN PINEAPPLE PLANTS 14 MONTHS OLD, ANALYZED IN DECEMBER, WITH 500 POUNDS OF NITROGEN, AS AMMONIUM SULPHATE, AND VARIABLE AMOUNTS OF K_2O FROM POTASSIUM SULPHATE, AS INDICATED BELOW.

Plant organs	Treatments		Amounts (grams) in total stem or leaves per plant									
	Symbols	K_2O lbs/A	Weights		Dry matter g.	Total sugars g.	Reduc. sugars g.	Sucrose g.	Starch g.	Total N. g.	Protein N. g.	Soluble organic N. g.
			Grams	Ratios								
Stems	X	0	333	1.00	59	6.2	4.7	1.5	23	1.1	.46	.65
	A	200	526	1.58	66	7.9	6.2	1.7	16	1.6	.55	1.05
	B	400	624	1.88	78	8.8	6.9	1.9	21	1.9	.67	1.23
	C	800	685	2.06	89	9.0	6.2	2.8	26	2.1	.70	1.40
	D	1600	852	2.56	104	9.3	6.1	3.2	30	2.6	.85	1.75
	E	3200	800	2.40	93	6.5	3.8	2.7	23	2.4	.81	1.59
Leaves	X	0	3465	1.00	576	99	83	16	13	12	7.8	4.2
	A	200	4250	1.23	608	96	68	28	8	14	8.1	5.9
	B	400	5200	1.50	816	104	73	31	15	17	12.2	4.8
	C	800	5410	1.57	844	108	76	32	14	17	11.7	5.3
	D	1600	6380	1.84	1015	121	83	38	23	19	12.9	6.1
	E	3200	5840	1.69	930	105	64	41	20	18	12.4	5.6

lute amounts of reducing sugars or starch and stem weights lacked statistical significance, presumably due to variations in the rates of mutual interconversion of these substances and to the small number of samples. In the leaves, similar correlations were statistically significant for dry matter, sucrose, total nitrogen and protein-N, with coefficients, r, of 0.98, 0.98, 0.98, and 0.96, respectively, at P = .01, and for total sugars, starch and soluble nitrogen, with correlation coefficients, r, of 0.78, 0.79, and 0.76, respectively, at P = .05.

The ratios of the weights of the stems or of the leaves of treatments E, D, C, B, or A, with 3200, 1600, 800, 400, or 200 pounds of added K_2O per acre, respectively, to similar weights of the X treatment, without added K_2O, in table I, increased with greater amounts of K_2O per treatment. Comparisons between the ratios of stem and leaf weights for similar K_2O treatments show that the former increased at greater rates than the latter. For instance, ratios of stem or leaf weights of the E, with 3200 pounds K_2O, to X, without added K_2O, was 2.40 for the stem and 1.69 for the leaves. Similar ratios for the stem or leaf weights of treatments E, D, C, B, or A to X were greater for the stem than the leaves, definitely indicating that high potassium increased stem weights more than leaf weights. Similar results were also obtained for the nutrient solution cultures under greenhouse conditions.

The data in figure 6, depicting concentrations of different substances in the basal, no. 1, and in the chlorophyllous distal, no. 4, sections of the active D leaves and in the apical stem sections for the various K_2O treatments of 14-months-old plants harvested in December, reveal results as follows:

In figure 6 A, nitrates in the basal leaf tissues of cultures with equal amounts of ammonium sulphate but different amounts of potassium sulphate increased with greater amounts of potassium, indicating that this element accelerated the intake, translocation and possibly assimilation of nitrate.

Phosphate concentrations, in figure 6 A, slightly lower in the cultures with great than small amounts of potassium sulphate, might have resulted from dilution effects due to greater plant weights in the former cultures. Total phosphate intake per plant, obtained by multiplying PO_4 concentrations by plant weights, was greater in the high- than low-potassium cultures.

Potassium intake correlated positively with the amounts of potassium supplied externally to the different treatments. The soil containing approximately 500 pounds of replaceable potassium per acre showed, in the X treatment, 2.4 mg. per gram of fresh tissue, approximately one half as high as that in treatment E, with 3200 pounds of added potassium.

In figure 6 B, soluble organic nitrogen and protein in the stem fluctuated slightly between the different treatments. However, in all treatments soluble organic nitrogen was from 1.80 to 2.50 times as great as protein nitrogen.

In the chlorophyllous distal, no. 4, sections of the leaves, soluble organic nitrogen and protein nitrogen fluctuated from 10 to 20% of total between different treatments.

Contrasting soluble organic and protein nitrogen in figure 6 B, the data show that the former was twice as great in the stem as in the leaves, and the latter twice as great in the leaves as in the stem. The differences in the amounts of these nitrogenous fractions between stem and leaves should be attributed to differences in the physiological functions of the tissues; the leaves requiring more protein-N in association with chlorophyll (**11**) than the stem, which is lacking in chlorophyll.

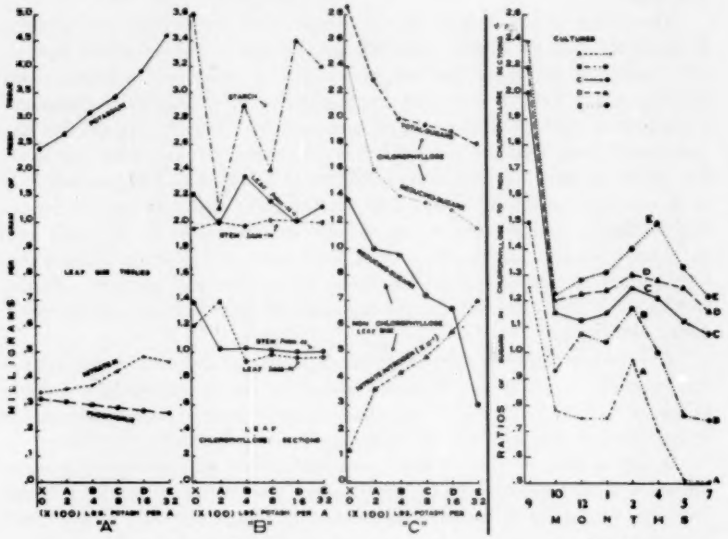

FIG. 6

FIG. 7

FIG. 6. Milligrams per gram of fresh tissue: (A), phosphate, nitrate, and potassium; (B), soluble organic nitrogen in the leaves and stem, or protein nitrogen in the leaves and stem; starch, total and reducing sugars; (C), sugars and nitrates in plants 14 months old, sampled in December, grown in the soil with 500 pounds of nitrogen as ammonium sulphate and 0, 2, 4, 8, 16, and 32 hundred pounds of K_2O as K_2SO_4 per acre.

FIG. 7. Ratios of sugar concentrations of the chlorophyllous medial sections to the non-chlorophyllous basal sections of the active (D) leaves of plants grown in solution cultures A, B, C, D, and E.

Starch, a product of sugar polymerization and in a state of constant translocation from the leaves to the stem, fluctuated considerably with maximal values at the X and D treatments. Starch concentrations may vary, depending on the amounts of sugars in excess of those required for growth and other urgent vital functions.

Treatment X, without added K_2O, showed greater concentrations of starch than all other treatments, which should be attributed to higher rates of sugar polymerization as a result of lower rates of sugar consumption in

growth. However, starch concentrations in all other treatments except in the 800 pounds of K correlated positively with amounts of potassium and negatively with sugars.

Total and reducing sugars, in figure 6 B, decreased with greater amounts of potassium, indicating greater rates of utilization in growth for the high than low potassium treatments.

Also, results obtained on sugar concentrations in the basal sections of the active D leaves, under greenhouse conditions, showed greater sugar concentrations for the low than high potassium cultures, which agree with the data obtained for the field grown plants.

The curves for the nitrate and reducing sugar concentrations in the basal sections of the active D leaves, in figure 6 C, showed more nitrate for the treatments with high than low potassium, while the curves for the concentrations of sugars were reversed in relation to potassium. The concentrations of reducing sugars in the tissues correlated negatively with the concentrations of nitrates with a coefficient, r, – 0.94, statistically significant at P = .01. These results indicate that the rates of sugar utilization were greater in the presence of high than low nitrates and potassium in the tissues.

The soil in which the plants were grown, of laterite type, ranged in replaceable potassium from 300 to 500 pounds per acre. It was fumigated before planting with chloropicrin at the rate of 150 pounds per acre by injection in the planting holes through the mulch paper. Rainfall during the last quarter of 1943, the time of planting, was 3.5 inches; for the entire year of 1944, the growing period of the plants, it was 29.5 inches. The rainfall for the first six months of 1945, the period of floral differentiation and fruit growth, was 22.7 inches. Average day temperature was 75 and 74 degrees, and night temperature 68 degrees for 1944 and 1945, respectively. Average light intensity, in foot-candles, was 4000 and 4100 for 1944 and 1945, respectively. The altitude of the field where the experiment was located is approximately 1000 feet.

These results are of a basic nature, and no attempt is made at this time to apply them to routine field operations, due to great variations in soil fertility and climatic conditions in different locations and for different years.

Discussion

The various data pertaining to the effects of time on the chemical composition of the leaves at different growth intervals, reveal that, with advancing physiological age, total nitrogen and potassium increased in the chlorophyllous, no. 4, but decreased in the non-chlorophyllous basal leaf sections. Calcium concentrations increased in the basal and chlorophyllous leaf sections with advancing age, with the exception of cultures F and G, with very low calcium in the nutrient solutions.

In the stem, calcium decreased with advancing plant age, except in culture E, with very great calcium concentrations in the nutrient solutions.

Calcium concentrations in the stem correlated inversely with potassium concentrations, with a coefficient, r, – 0.84, statistically significant at P = .01.

Much greater accumulations of calcium have been observed in the stems of preflowering than of postflowering plants, presumably resulting from differences in the rates of calcium movement from the stem to the leaves. Such differences in calcium concentrations were explained on the basis of differences in the anatomy of the apical tissues of the stem of preflowering and postflowering plants. In preflowering plants, the apical tissues of the stem, with undifferentiated meristematic tissues and with many conducting vessels incompletely developed, the movement of calcium from the stem to the leaves was not as rapid as through the stems of postflowering plants, the latter with all conducting vessels completely developed. KRAUSS (**3**), in a diagrammatic illustration of the apical meristem of preflowering plants, in her figure 32, showed three distinct areas, namely, the undifferentiated acroscopical, the differentiating but unspecialized, and the specialized meristematic tissue which, due to differences in the development of the conducting vessels at different stages of plant growth, may vary considerably in degree of permeability to ions or molecules of different elements, the rates of conduction or of translocation increasing with tissue maturity and completion of the development of the vessels, and *vice versa*.

The movement of ions or molecules through meristematic tissues, lacking conducting vessels, may be subject to forces related to osmosis, surface tension or water movement by transpiration, in which case ions with small radii, such as potassium, may enter the tissues more readily than ions with large radii, such as calcium (**2**).

Sugar fluctuations in the non-chlorophyllous basal sections of leaves, of meristematic status, of cultures with variable nitrate concentrations, at different growth intervals, were attributed to different rates of utilization in growth. Similar fluctuations in the chlorophyllous distal sections were attributed to variations in photosynthetic efficacy.

During and following the period of flowering, the stem and leaves of the pineapple plant attain little or no additional growth. With the cessation of growth in the tissues of the basal sections and gradual senescence in the tissues of the chlorophyllous distal sections, sugar concentrations in the leaves may be decreased by translocation to the fruit, slips, suckers, and roots for the promotion of growth activities, although similar activities in the basal sections of the leaves already had ceased.

Sugars utilized for growth in the meristematic tissues of the basal regions of the active D leaves are translocated from the chlorophyllous distal sections of the same leaves wherein they are produced. Very young leaves, lacking ample areas of chlorophyllous tissues for the synthesis of sugars required in the apical meristematic regions of the stem, are supplied with sugars translocated from mature leaves or from reserves in the stem.

In view of the close association of the sugar supplies produced by the chlorophyllous tissues of the distal sections with those consumed for growth

by the meristematic tissues of the non-chlorophyllous basal sections of active leaves, the curves, in figure 7, depicting the sugar ratios of the chlorophyllous distal to the non-chlorophyllous basal sections at different growth intervals, suggest that potential sugar availability varied under the conditions of the different cultures. For example, the curve for culture A, with very low nitrate, and that of B, with twice as much nitrate, both with suboptimal supplies of nitrate, indicated low ratios, suggesting that sugar utilization was limited by inadequate nitrate supplies. The curves for cultures C, D, and E, occupying higher levels, indicated greater supplies of sugars for utilization than for A and B, presumably due to greater photosynthetic efficacy in the high nitrate cultures.

Turning figure 7 upside down, thereby reversing the interpretation of the sugar ratios from "consumption" to "accumulation," we observe that the curves for "accumulation" of sugars in the meristematic tissues of the basal sections were greater in the cultures A and B, with lower nitrate concentrations in the nutrient solutions, than in cultures C, D, and E, with higher concentrations of nitrate.

The ratios of sugar concentrations in the chlorophyllous distal to the non-chlorophyllous basal sections may be employed as a basis for the evaluation of sugar utilization in growth under differential nitrogen nutrition, although its use may be limited in application under conditions involving other variables of mineral nutrients than those in the experiments under discussion. However, with additional experimentation, a more precise method than the present may be evolved for the interpretation of the sugar ratios and their meaning in plant growth.

The biochemical and physiological processes involved in the utilization of sugars for growth, *via* fermentation by phosphorylation or other paths of oxidation, have been reviewed by BARRON (**1**) and Krebs (**4**).

Experimental results of the authors (**12**) indicated that the leaves of pineapple plants accumulated at night, in darkness, great quantities of di- or tri-carboxylic acids, *i.e.*, malic or citric, which disappeared during the day, in light. The rates of organic acid accumulation increased with higher growth rates and vice versa. Moreover, plants with high growth rates contained smaller amounts of sugars than with low rates, suggesting that sugars were oxidized to malic or citric acid in the metabolic processes for growth. The disappearance of malic and citric acids in the leaves during daylight could not be definitely traced in other products of metabolism, although there was a slight indication of combination with nitrogen and conversion to amide or amino compounds.

THIMANN and his colleagues (**14, 15**) observed that the 4-carbon acids, *i.e.*, malic, etc., accelerated the rates of growth, respiration, and protoplasmic streaming in the presence of auxin, sugar, and oxygen. The same author compared the action of auxin to that of a switch causing the metabolism of carbohydrate, especially of pyruvate, to go *via* the growth-promoting system instead of by another. Auxin determinations in the stem and leaves of Cabezona pineapples, by VAN OVERBEEK *et al.* (**16**), showed concentra-

tions of bound auxin at 1.0 and 11.0 γ and of free auxin at 6.1 and 0.8 γ per gram of tissue for the apical stem and basal sections of young leaves, respectively. In the highly differentiated areas of the leaves, lacking meristematic tissues, both auxin fractions were very small, ranging from 1.0 to 0.7 γ.

The studies by VAN OVERBEEK *et al.* (**16**), THIMANN *et al.* (**14, 15**), and the authors (**12**), associating the metabolic processes of growth with 4-carbon carboxylic acids, sugars and auxin, present a group of inter-related data, illuminating certain of the pathways leading to growth.

Summary

The results obtained at different growth intervals on the chemical composition of the leaves and stems of pineapple plants grown in solution cultures and in the soil with different amounts of nitrate, potassium and calcium were, as follows:

1. Total nitrogen in the non-chlorophyllous basal sections of leaves, of meristematic status, and in the stem, correlated positively with the concentrations of nitrate in the nutrient solution, and was greater in the preflowering than postflowering stages of plant growth. In the chlorophyllous distal sections of the leaves, total nitrogen correlated with the nitrate concentrations in the nutrient solutions, but was greater in the postflowering than preflowering growth periods.

2. Potassium in the non-chlorophyllous basal and in the chlorophyllous distal sections of the leaves correlated positively with the concentrations of potassium and negatively with those of calcium in the nutrient solutions. In the non-chlorophyllous basal sections of the leaves and in the stem, potassium concentrations were greater in the preflowering than postflowering stages, but in the chlorophyllous distal sections similar concentrations were reversed.

3. Calcium in the non-chlorophyllous basal and chlorophyllous distal sections of the leaves correlated positively with the concentrations of the same in the nutrient solutions. Calcium concentrations were almost twice as great in the stem as in the leaves, and much greater in the preflowering stage, in the stems, due to the retarded movements of calcium from stem to leaves through the incompletely developed conducting vessels in the meristematic tissues, than in the postflowering stage, with well developed vessels.

4. Sugar concentrations in the non-chlorophyllous basal sections were greater in the low- than high-nitrate cultures. Also, sugar concentrations were lower at the preflowering than postflowering periods. In the chlorophyllous distal sections, sugar concentrations were greater in the high nitrate cultures of the series with variable calcium and constant potassium, but in the series with variable potassium and constant calcium, they were greater in the low nitrate cultures. The results indicate that sugars increased in the latter series due to decreased plant growth resulting from the combined effects of low nitrate and potassium concentrations.

In the stem, differences in sugar concentrations between different cultures were not as great and distinct as in the leaves, except in the cultures with low concentrations of potassium and nitrate where the sugars were much greater than in the cultures with high concentrations of potassium and nitrate.

5. In field grown plants, sugar concentrations in the non-chlorophyllous basal leaf sections correlated inversely with the nitrate and potassium concentrations of the tissues and with growth.

6. Absolute amounts of sugars or nitrogen per plant increased with greater plant weights, although the concentrations of these substances decreased at rates slightly lower than those of growth, as measured by plant weights.

7. Ratios of sugar concentrations in the chlorophyllous distal sections to the non-chlorophyllous basal sections of the active leaves, indicating potential sugar supplies available to the meristematic tissues of the basal leaf sections for utilization in growth, were greater for the high nitrate cultures and correlated positively with plant growth.

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EXPERIMENTS ON THE INHERITANCE OF NIACIN IN CORN (MAIZE)[1]

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Introduction

In an earlier publication the writers (**12**) showed that the niacin concentration of dent corn behaved as a so-called quantitative character in inheritance, being determined by many genes of small individual effects. They also showed that niacin concentration could be modified rapidly by selection and saw no reason why hybrids with niacin concentrations as high as 50 micrograms/gram could not be developed. It was noted further that a second mode of inheritance had been reported (**1, 9**). In this, the starchy, *Su*, gene was dominant for lower niacin concentration over its *su* allele, just as it is dominant for starchy vs. sweet endosperm. We suggested that the influence of the *Su su* alleles on niacin concentration was a secondary or pleiotropic one. There also was a suggestion (inconclusive) that the alleles differentiating starchy and waxy (*Wx wx*) might operate similarly.

Since our paper was written, workers at the Ohio Experiment Station have reported on niacin concentration in dent corn. Their earlier results (**7**) are open to some question because their material was open pollinated. Pollination accordingly could have been a differentiating influence, rather than environment as they concluded. Their later research was with hand-pollinated material. From this (**4**), they stressed the hereditary influence on niacin concentration, and suggested that it might be increased by breeding. GORFINKEL (**5**) also has reported practically complete dominance of the *Su* gene for lower niacin, and CAMERON and TEAS (**3**) have found the sugary types as conditioned at both the *su* and the *du* loci to be higher in niacin than the starchy types. LENG *et al.* (**8**) report the niacin concentration of sugary, waxy and dent kernels from segregating ears. Dent and waxy are not mutually exclusive, and neither are sugary and waxy. Their sugary class included the double recessive *su wx* kernels; whether or not their dent included waxy as well as starchy cannot be told. Correlations reported indicate a multiple effect of the *su* gene on both niacin concentration and carbohydrate formation, a pleiotropic action as postulated by RICHEY and DAWSON (**12**). LENG *et al.* (**8**) also state that unpublished data indicate no close relation between kernel size and niacin concentration, in agreement with the conclusion previously reported by RICHEY and DAWSON (**12**).

[1] Joint contribution from the Division of Cereal Crops and Diseases, Bureau of Plant Industry, Soils, and Agricultural Engineering, Agricultural Research Administration, U. S. Department of Agriculture; the Agricultural Experiment Station of the University of Tennessee; and the Department of Botany of Columbia University.

The interaction of multiple-factor with *Su su* hereditary control seemed of interest in various ways. Particularly, it appeared to offer another approach for the study of physiogenetic relations. Experiments accordingly were undertaken to explore the situation further. An initial result was to rule out the greater weight of starchy kernels as any important part of the reason for their having a lower niacin concentration than that of their sweet sibs. A parenthetic note of this was added in proof to our earlier paper (**12**). Progress with these and certain corollary experiments is reported here.

Material and methods

The material investigated can be considered most conveniently in connection with the particular experiments. All values for niacin reported originally here have been obtained by modifications of the procedures recommended by the ASSOCIATION OF VITAMIN CHEMISTS (**13**). *Lactobacillus arabinosus* (ATCC 8014) was the test organism. Evidence will be presented elsewhere to show that absolute limitations to the rate of acid production are inherent in the Vitamin Chemists' procedure as published. MCILWAIN (**10**) has pointed out that such limitations both theoretically and practically render the assay results somewhat unreliable. These difficulties have been partially avoided in the present study by the following changes in procedure.

1) An operationally induced deficiency of cystine was overcome by standardization of the method of mixing the ingredients of the basal medium. Precipitation of cystine was avoided by adding the stock solution of this amino acid to the maximum allowable volume of water at 50° C.

2) The concentration of glucose was increased to 1.2%.

3) Potassium acetate replaced sodium acetate and the concentration was increased to 1.5%.

4) Final pH of the basal medium was lowered to 6.4.

5) All bacterial cultures, inocula and sterilized media were stored whenever necessary in a refrigerator in which no possibility existed for contamination with vapors of chloroform, toluene or other volatile chemicals. The importance of this precaution needs especial emphasis in view of the recommendation of the VITAMIN CHEMISTS (**13**).

6) All acid extracts of whole corn meal were diluted 1 part to 1000 of water, based on the dry weight of the original sample, before withdrawal of aliquots for assay.

As a consequence of the first five of these modifications, the linear portion of the standard curve attains a virtually constant slope of four millimoles of acid produced per microgram of added nicotinic acid in a period of 65 hours.

Comparison of the assay values obtained by the Vitamin Chemists' method (**13**) with those obtained on parallel samples by the modified procedure outlined above indicates that all previously published figures from this Laboratory (**12**) are about 2.5 micrograms per gram too high. This

appears to have been caused by partial alleviation of growth factor deficiencies (*e.g.*, cystine) other than niacin upon the addition of corn meal extracts to the basal medium. In consequence, the standard curves were uniformly a little too low.

Standard samples of corn meal and of wheat flour were introduced at intervals during the course of the assays here reported, always with satisfactory results.

In some of our studies, there arose the problem of possible deterioration of niacin content during storage of the samples. Experiments performed on whole grain and on ground meal, both of which had been stored at room temperature for two years, indicated that no significant deterioration had occurred in the whole grain. On the other hand, the ground meal had lost about 3.0 micrograms of niacin per gram, a negligible amount for most purposes.

The question of completeness of niacin extraction from the ground whole meal has also arisen. The intermediate model of the Wiley Mill (A. H. Thomas Co., Philadelphia, makers) has been used to grind the corn samples to meal following preliminary cracking in a plate mill. There has been a tendency for the hard portions of the grain to remain behind in the grinding chamber of this mill when a 40- or 60-mesh screen is used. Careful and repeated tests of completeness of niacin extraction from samples specially ground through 20- and 40-mesh screens to avoid hold-up have shown complete recovery of the vitamin from the 20-mesh sample. Hence, we have adopted the 20-mesh sieve as standard.

Niacin in normal and defective seeds

A possible influence of kernel size on niacin concentration was explored by the assay of normal (*De*) and small or defective (*de*) seeds. The defective character was derived from an ear of the Huffman variety first selfed in 1943. Normal seeds from segregating ears were planted in 1944 and 1945, and both normal and defective seeds were planted in 1946. The plants from defective seeds developed more slowly, requiring about seven days longer from planting to silking. At that time they were about eight feet tall compared to nine feet for the normals. Two selfed segregating ears from this crop had 440 and 521 kernels, of which 107 and 126 were defective—deviations of three and four from a 1 to 3 ratio. In 1947, selfed and crossed ears were obtained, as shown in table I, and the two kinds of seeds assayed for niacin. These should have been very largely isogenic except at the *De de* locus. Considering first only the normal and defective seeds produced on individual segregating ears, the normal seeds weighed 15.2 grams or 69% more than the defectives and had 2.58 ± 0.11 micrograms or 57% more niacin per seed. The concentration of niacin in the defective seeds was 1.3 ± 1.2 micrograms per gram, or 7%, greater than that in the normal seeds. This difference is not significant statistically and relatively unimportant in any event. The data for one homozygous normal

and two homozygous defective plants are from too small a sample to be conclusive as to anything. They suggest in no way, however, any important modification of niacin concentration when the normal and defective seeds are grown on different plants instead of in direct competition on the same ear. There is nothing here, then, to indicate that even large differences in kernel size modify importantly the niacin concentration per gram of dry matter.

The relative influence of seed and pollen parents

It was noted in our earlier paper that the seed parent in general exercised more influence than the pollen parent. It seemed advisable to have a quantitative estimate of the relative influence of the two parents. Such a differential influence has been noted for several kernel characteristics in

TABLE I

DATA ON NIACIN CONTENT OF NORMAL (*De*) AND DEFECTIVE (*de*) SEEDS OF HUFFMAN LINE H6-2-2 IN 1947.

Kind of mating	Weight of 100 seeds grams		Niacin content in micrograms			
			Per gram		Per seed	
	De	*de*	*De*	*de*	*De*	*de*
de de × self		18.0		21.4		3.85
de de × self		18.3		28.1		5.13
Average		18.2		24.8		4.49
De De × self	28.6		18.9		5.41	
De de × self	27.6	15.5	22.5	20.0	6.21	3.10
De de × *de de*	38.5	23.5	17.0	18.7	6.55	4.39
De de × *de de*	34.2	23.5	18.2	18.4	6.22	4.32
De de × *de de*	43.8	25.0	17.2	21.6	7.53	5.40
de de × *De de*	41.4	22.2	21.7	24.3	8.98	5.39
Average*	37.1	21.9	19.3	20.6	7.10	4.52

*Normal and defective seeds from same ears.

corn, perhaps the first being in floury-flinty endosperm (**6**). In crosses between these two types, the seed parent determines the nature of the endosperm, the two genomes from the polar nuclei overbalancing the one genome from the sperm in the triple fusion of endosperm initiation. Niacin in the corn kernel is not restricted to the endosperm. It occurs also in the germ (embryo plus scutellum) and pericarp, more or less equally with that in the endosperm (**12**). The endosperm, however, comprises about 90% of the kernel. Any differential influence of the parents on niacin in the endosperm accordingly would very largely determine difference in the content of the whole kernels. It also is possible, of course, that niacin concentration could be influenced by the physiology of the seed-parent plant, apart from the genetic influence of this plant directly on the endosperm.

TABLE II

THE NIACIN IN T13, Tx61M AND THEIR RECIPROCAL F_1 CROSSES, CROP OF 1947, AND THE CALCULATED NIACIN IN THE CROSSES BASED ON AN ASSUMED INFLUENCE OF SEED AND POLLEN PARENTS IN A RATIO OF 2 TO 1.

Material	Micrograms per					
	Seed			Gram		
	Observed	Calc'd	Dif.	Observed	Calc'd	Dif.
T13	15.07	....	....	55.8	....	....
Tx61M	5.54	....	....	21.3	....	....
T13 × Tx61M	11.77	11.89	0.12	32.7	44.3	11.6
Tx61M × T13	8.12	8.72	.60	24.6	32.8	8.2

Crosses between T13 and Tx61M were used to study the relative influence of seed and pollen parents on niacin concentration. These are long-time (10 or more generations) selfed lines and differ widely in niacin concentration. They were also used, moreover, in the investigation of niacin in crosses between sweet and starchy corn to be reported here. The inbreds were crossed reciprocally and the crosses were selfed, and also backcrossed by both parents. The niacin in the parents and single crosses grown in 1947 is shown in table II. The calculated values are based on the parental concentrations and an assumed ratio of an influence of two for the seed parent to one for the pollen parent. The agreement between observed and calculated niacin per seed is excellent, but the calculated niacin per gram is excessive. The excesses for the two reciprocals, however, differ by only 3.4 micrograms per gram.

The single cross between T13 and Tx61M and both parents were grown in adjacent rows and self-pollinated in 1948. Crossed plants also were backcrossed with pollen from each parent. The niacin per seed and per gram in the resulting kernels is shown in table III. As the concentration of niacin for the parent lines in 1948 was slightly lower than that for 1947, the 1948

TABLE III

THE NIACIN IN T13, Tx61M AND CERTAIN PROGENIES DERIVED FROM THEM IN 1948, AND THE CALCULATED NIACIN BASED ON AN INFLUENCE OF SEED AND POLLEN PARENTS IN A RATIO OF 2 TO 1.

Material	No. of ears	Niacin in micrograms					
		Per seed			Per gram		
		Observed*	Calculated	Dev.	Observed*	Calculated	Dev.
T13 × Self	3	20.8 ± 1.6	....	....	51.2 ± 1.7	....	....
Tx61M × Self	5	5.9 ± 0.1	....	....	17.9 ± 0.5	....	....
(T13 × Tx61M), F_2	3	12.3 ± 0.5	13.4	1.1	27.5 ± 1.7	34.6	7.1
do ×T13	3	13.4 ± 0.4	15.8	2.4	31.7 ± 1.5	40.1	8.4
do ×Tx61M	3	8.8 ± 0.3	10.9	2.1	20.6 ± 1.0	29.0	8.4

*The values following the ± are the standard errors for the averages shown.

values were used to get the calculated values in table III, as those for 1947 had been used for calculation in table II. It will be noted that the niacin observed is consistently less than that calculated, both per seed and per gram. The assumption of a ratio of two for the influence of the seed parent to one for that of the pollen parent appears justified as a reasonably approximate basis for estimating differences between the reciprocals of T13 with Tx61M. The absolute concentrations, however, were consistently lower than those calculated from the parental values.

No reason is known why the observed concentrations in the F_1 and F_2 crosses and in the backcrosses should be consistently lower than the calculated values based on parental concentrations. Differences in kernel size between the parents and the F_1 crosses would be of negligible importance in relation to the size of the deficits. Even the excess size of the F_2 and

TABLE IV

THE NIACIN CONCENTRATION (MICROGRAMS PER GRAM) OBSERVED IN THE SEED OF 12 F_1 CORN HYBRIDS AND THEIR RECIPROCALS, AND THE CALCULATED CONCENTRATION BASED ON AN INFLUENCE OF SEED TO POLLEN PARENT OF 2 TO 1. (COMPUTED FROM RICHEY AND DAWSON (12), TABLE VIII.)

Hybrid	As in column 1			Reciprocal			Av. dev.
	Obs.	Calc.	Dev.	Obs.	Calc.	Dev.	
T202 × Cl.7	23.3	20.6	2.7	23.7	21.5	2.2	2.45
× Cl.21	21.5	23.3	−1.8	30.9	27.1	3.8	1.00
× T8	21.4	20.3	1.1	20.5	21.0	− .5	.30
× K4	20.7	18.3	2.4	16.0	16.9	− .9	.75
× Ky35–7	23.2	20.8	2.4	21.2	22.0	− .8	.80
× KYS	20.2	20.5	− .3	21.2	21.4	− .2	.25
Cl.7 × Cl.21	19.2	25.3	−6.1	34.0	28.0	6.0	− .05
× T8	21.2	22.2	−1.0	....	....	....	....
× KYS	20.4	22.4	−2.0	19.1	22.4	−3.3	−2.65
T8 × K4	21.2	19.7	1.5	16.9	17.6	− .7	.40
× KYS	24.8	21.9	2.9	21.4	22.1	− .7	1.10
K4 × KYS	19.7	17.8	1.9	22.2	20.1	2.1	2.00

backcrossed kernels over that of the inbreds would be much smaller than that of the normal over the defective seeds, which had approximately equal niacin concentrations. Moreover, there is also a deficiency in the niacin per seed. Finally, there is no such deficiency of observed below calculated concentration in many other crosses. Thus, the observed and calculated niacin concentrations of 12 single crosses and 11 of their reciprocals are shown in table IV. These were computed in the same way from data in table VIII of RICHEY and DAWSON (12). The deficiencies and excesses largely balance in size and frequency, with a mean that is slightly positive, not negative as in the crosses of T13 and Tx61M. Data for the concentrations of five F_2 crosses also are shown in the 1948 table VIII. In spite of the larger kernels in such material, there were three positive to two negative deviations of observed from calculated concentration. The reciprocal crosses between

T13 and T61 (T61 is not related to Tx61M), however, had deficiencies of 4.0 and 5.0 below those calculated from the parents. Possibly, then, this tendency is associated with T13. A gene or complex recessive for high niacin would result this way. It is the mode of action for the *Su su* locus; but there the effect of *su* on niacin can be identified by its pleiotropic effect in determining sugary kernels. However that may be, the ratio of two to one again appears to provide a substantially suitable basis for seed and pollen parent influence.

Niacin concentration in sweet-starchy hybrids

In so far as possible, the starchy (*Su*) inbreds, T13 and Tx61M, were crossed reciprocally with six sweet corn inbreds in 1947. The latter were well established lines of 10 or more generations of inbreeding. They are poorly adapted as far south as Tennessee, however, and differ from T13 and Tx61M by about 20 to 25 days in the time required from planting to flowering. Consequently, several of the desired crosses were missed. The niacin concentration of those that were obtained and of the parent inbreds is shown in table V. The values for T13 and Tx61M are the same as in table II, and are assays of the bulked seed of these inbreds grown in 1947. The assays of the sweet inbreds as grown in 1947 and 1948 are both shown because of the extreme discrepancies for three of the six lines. The seed of P14, P39 and 39K2 was developed very poorly in 1947 and it was thought that this might account for the higher niacin in that year. To check on this, Mr. Glenn Smith of the Purdue Agricultural Experiment Station kindly grew

TABLE V

THE NIACIN CONCENTRATION (MICROGRAMS PER GRAM) OF SOME SWEET CORN INBREDS AS GROWN IN TENNESSEE IN 1947 AND 1948, AND IN INDIANA IN 1949, OF STARCHY INBREDS T13 AND Tx61M IN 1947 AND OF F_1 SEED OF THE RECIPROCAL STARCHY-SWEET CROSSES IN 1947. THE CALCULATED (CALC.) CONCENTRATIONS WERE OBTAINED AS DESCRIBED IN THE TEXT.

Name	Sweet corn inbred parent			Starchy inbred parent*							
	Grown in			T13, used as:				Tx61M, used as:			
	Tennessee		Indiana 1949	Female		Male		Female		Male	
	1947	1948		Obs.	Calc.	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
P14**	46.7	29.9	36.3 ± 3.3	35.6	44.2	24.0	32.6	16.6	23.6	18.9	25.9
P39	59.1	30.5	33.2	32.3	....	....	....	....	....	....	....
39K2**	43.0	34.6 ± 0.3	28.8 ± 2.4	35.0	42.5	21.7	29.2	18.8	21.4	18.9	21.5
51K2	59.1	57.1 ± 3.2	56.0 ± 3.0	42.9	....	....	....	15.0	....	22.7	....
I45**	27.7	26.2 ± 0.6	24.4 ± 1.3	38.3	44.3	26.8	32.8	20.9	16.3	15.9	11.3
G192	29.7	29.6	.....	33.6	....	....	....	....	....	....	....
Av.**	39.1	30.2	29.8	36.3	43.7	24.2	31.6	18.8	20.6	17.9	19.8

*The niacin concentration of the starchy inbreds was (1947) 55.8 for T13 and 21.3 for Tx61M.

**The averages for the three inbreds so marked and their hybrids.

selfed seed of five of the inbreds at Lafayette, Indiana, in 1949. The niacin concentrations of these are also shown in table V. The value for P39 is based on the only ear obtained. The others are the means for three ears. They are definitely more in line with the Tennessee 1948 data. That the starchy kernels from the crosses of I45 and G192 with T13 contained higher concentrations of niacin than the sugary parents makes is obvious that all crosses of sweet with starchy need not have a lower niacin concentration than the sweet parent. The situation is more complex and depends upon the particular material used.

In addition to the niacin concentrations observed in the different crosses, the concentrations as calculated from those of the parents are shown in table V for the three sweet inbreds having reciprocal crosses with both T13 and Tx61M. These calculations are based on an assumption that each sweet inbred has an inherent niacin concentration apart from the influence of the *su* gene. In crosses with starchy corn, the effect of *su* will be suppressed by its *Su* allele, and the niacin concentration of the cross will be determined by this inherent concentration of the sweet parent and the effective concentration of the starchy parent. In the absence of other information, this "effective concentration" will be taken as that determined by assay. But it must be recognized that other genes could act on niacin similarly to *su*, as already suggested. If so, the starchy corn could also have an inherent concentration which would be the only one effective in F_1 crosses.

The method of calculating the niacin concentrations for the averages of the reciprocals of the three sweets with T13 is used as an example. The inherent concentration of the sweets will be symbolized by L.

$$\begin{aligned} T13 \times su, 36.3 &= \tfrac{1}{3}[(2 \times 55.8) + L] \\ su \times T13, 24.2 &= \tfrac{1}{3}[(1 \times 55.8) + 2L] \\ \text{Difference, } 12.1 &= \tfrac{1}{3}(55.8 - L) \end{aligned}$$

$L = 55.8 - (3 \times 12.1) = 19.5$, the inherent niacin concentration for the average of three sweets in crosses with T13.

As a check, the calculated concentration for the average of the crosses then becomes:

$$\begin{aligned} T13 \times su &= \tfrac{1}{3}[(2 \times 55.8) + 19.5] = 43.7 \\ su \times T13 &= \tfrac{1}{3}(55.8 + 39.0) = 31.6 \\ &\text{Difference} = 12.1 \end{aligned}$$

The values for the individual crosses of P14, 39K2 and I45 were calculated similarly and the corresponding values for crosses with Tx61M were computed using its 21.3 concentration instead of the 55.8 concentration of T13.

The calculated values for the crosses with T13 are 8.6, 7.5 and 6.0 micrograms per gram above the observed values, the excess for the average being 7.4. These are in reasonable agreement. The differences between observed and calculated concentrations for the Tx61M crosses are not in good indi-

vidual agreement, ranging from a plus of 7.0 to a minus of 4.6. The calculated and observed average values differ, however, by only 1.8 micrograms per gram, in contrast to the difference of 7.4 for the T13 crosses. The inherent concentration for the three sweet corns estimated from their crosses with Tx61M is 18.6, to compare with that of 19.5 based on the T13 crosses. Considering that the observed values of T13 and Tx61M used as bases in calculating the two estimates differed by 34.5 micrograms per gram, this seems highly satisfactory.

The five comparisons between observed and calculated niacin concentrations in the crosses and backcrosses of T13 and Tx61M (tables II and III) showed excesses for the calculated of 11.6, 8.2, 7.1, 8.4, and 8.4 micrograms per gram. Combining these with the 7.4 excess from table V, gives an average of 8.5 ± 0.7 micrograms per gram as the average excess of the calcu-

TABLE VI

THE NIACIN CONCENTRATIONS OF CERTAIN CORN CROSSES INVOLVING INBRED T13: (a) AS ORIGINALLY CALCULATED AND SHOWN IN THE TABLE STATED, (b) AS MODIFIED BY SUBTRACTING 8.5 FROM EACH, AND (c) AS OBSERVED, AND THE DIFFERENCES (b)–(c).

Table	Type of cross	Niacin (micrograms per gram)			
		Calculated		Observed	Difference
		Original	−8.5		
2	T13 × Tx61M	44.3	35.8	32.7	3.1
2	Tx61M × T13	32.8	24.3	24.6	− .3
3	(T13 × Tx61M) × S	34.6	26.1	27.5	−1.4
3	do × T13	40.1	31.6	31.7	− .1
3	do × Tx61M	29.0	20.5	20.6	− .1
5	T13 × *su**	43.7	35.2	36.3	−1.1
	All	37.4	28.9	28.9	0.0

* Average of three sweet corn crosses.

lated concentrations. The observed and calculated values for T13 crosses from tables II, III, and V are brought together in table VI, and the 8.5 average excess is deducted from each of the originally calculated values. There seems little doubt that the assay values of T13 are higher than the niacin concentration it transmits to its F_1 and F_2 crosses and its backcrosses with Tx61M. In other words, T13 appears to have an inherent or effective niacin value some 8.5 micrograms per gram less than its assay value, just as the inherent niacin of the sugary inbreds is lower than their assay values.

Second generation of the sweet-starchy hybrids

The F_1 sweet-starchy hybrids were grown in 1948, were backcrossed by and on the sweet parents, and were selfed. The assay values for the starchy and sugary kernels from the resulting ears are shown in table VII. For all

selfings and crossings of *Su su* × *su su*, the data are the averages for the individual assays of the numbers of ears stated. The same is true for the 51K2 progenies from matings of *su su* × *Su su;* for other progenies from this kind of mating the data are single assays of bulked samples from the numbers of ears stated. The standard deviations are based on variances among ears within individual progenies, and do not comprise any of the variation between progenies. They apply to the average concentrations shown immediately above them in the table. Thus, 0.68 is the standard deviation

TABLE VII

THE NIACIN CONCENTRATION (MICROGRAMS PER GRAM) OF STARCHY (*Su*) AND SWEET (*su*) KERNELS OF CORN FROM SEGREGATING EARS RESULTING FROM BACKCROSSING STARCHY-SWEET F_1 CROSSES BY AND ON THE SWEET PARENTS, AND FROM SELFING THE F_1 CROSSES.

Original starchy-sweet cross	Backcrossed *Su su* × *su su*				Backcrossed *su su* × *Su su*				*Su su* selfed			
	No. of ears	Kernel type			No. of ears*	Kernel type			No. of ears	Kernel type		
		Su	*su*	Dif.		*Su*	*su*	Dif.		*Su*	*su*	Dif.
Tx61M × P14	4	23.2	25.0	1.8	7	21.1	27.2	6.1	2	20.0	28.7	8.7
do × P39	4	16.3	23.8	7.5	6	20.8	37.5	16.7	1	17.8	25.5	7.7
do × 39K2	5	20.5	28.2	7.7	6	23.7	36.1	12.4	0	....	....	....
do × 51K2	7	22.4	40.1	17.7	5	22.5	45.8	23.3	4	23.0	37.8	14.8
Total or Av.	20	20.6	29.3	8.7	24	22.0	36.7	14.7	7	20.3	30.7	10.4
S. Dev. of Av.	....	0.68	1.02	0.55	5*	0.91	3.32	2.82	....	.44	1.66	1.52
T13 × P14	5	36.2	48.3	12.1	8	26.4	35.0	8.6	3	31.1	49.3	18.2
do × P39	5	25.6	43.0	17.4	4	25.5	49.0	23.5	2	34.5	58.0	23.5
do × 39K2	6	30.2	44.6	14.4	7	26.5	40.9	14.5	2	44.6	65.3	20.7
do × 51K2	4	31.8	64.2	32.4	3	26.0	51.1	25.1	1	41.5	85.1	43.6
Total or Av.	20	31.0	50.0	19.0	22	26.1	44.0	17.9	8	37.9	64.4	26.5
S. Dev. of Av.	....	0.79	0.92	0.48	3*	1.65	1.45	2.69	....	1.25	2.05	.88
T13 × I45	13	25.2	38.0	12.8	14	17.9	29.9	12.0	3	34.6	54.9	20.3
S. Dev.	....	1.02	1.22	0.72	....	....	....	....	....	4.00	3.66	1.90
General Av.	53	25.6	39.1	13.5	60	22.0	36.9	14.9	18	30.9	50.0	19.1
S. Dev. of Av.	....	0.47	0.60	0.33	....	....	....	....	....	0.98	1.28	0.79
C. V. per cent.		13.3	11.0	17.3	....	....	....	....	....	....	....	....

*The standard deviations so marked are for the 51K2 progenies only, the other ears of *su su* × *Su su* having been bulked by progenies before assay.

of the mean concentration of 20.6 in the starchy kernels from 20 ears of (Tx61M × *su*) × *su*.

The sweet kernels in every case carry a higher niacin concentration than the starchy kernels on the same ears. This excess niacin concentration, however, is not uniform for the different sweet inbreds used, nor for the two starchy parents with which they were crossed. As might have been expected, the segregates from crosses with T13 have a higher niacin concentration than do those involving Tx61M, for the sweet kernels as well as for the starchy ones. Moreover, the excess niacin in the sweet kernels above that in the comparable starchy kernels is greater in the T13 crosses.

No comparison can be made adequately of the two kinds of backcrosses from the data as shown in table VII. The starchy parent will have an influence on the endosperm of $\frac{1}{3}$ in the backcrosses on the heterozygous parent and of $\frac{1}{2}$ in the backcrosses on the sweet parent. The difference of some 30 micrograms per gram between Tx61M and T13 thus becomes the overshadowing influence. Evidence as to the possible influence of the parent plant, apart from the direct genetic influence in endosperm formation, however, can be had from the data in table VIII. These comprise estimates of the niacin contributing power of the different sweet corn inbreds in the different matings used. These estimates eliminate the differential influence of Tx61M and T13 on niacin concentration. The values within the Tx61M (22.0 vs. 22.9) and within the T13 (20.8 vs. 21.1) material for the backcrosses in which the heterozygous *Su su* plants and the homozygous *su*

TABLE VIII

THE NIACIN-CONTRIBUTING POWER OF FOUR SWEET CORN INBREDS, AS ESTIMATED FROM THE STARCHY SEEDS OF CROSSES WITH Tx61M AND T13 WHEN SELFED AND RECIPROCALLY BACKCROSSED BY AND ON THE SWEET PARENTS.

Sweet parent	Starchy parent				Averages for backcrosses involving			*Su su* selfed, when starchy parent was		
	Tx61M		T13							
	Su su × *su su*	*su su* × *Su su*	*Su su* × *su su*	*su su* × *Su su*	Tx61M	T13	Both	Tx61M	T13	Av.
P14	25.9	21.7	28.7	21.4	23.8	25.1	24.4	22.0	11.0	16.5
P39	15.5	21.4	12.8	20.4	18.5	16.6	17.5	17.7	17.8	17.8
39K2	21.8	24.9	19.7	21.6	23.4	20.7	22.0	....	38.0	....
51K2	24.7	23.4	22.1	21.0	24.1	21.6	22.8	28.0	31.8	29.9
Av.	22.0	22.9	20.8	21.1	22.4	21.0	21.7	22.6	20.2*	21.4

* Not including 38.0.

plants were used respectively as seed parents are in close agreement. This is excellent evidence that the inherent physiology of these two kinds of plants had no important influence on niacin concentration in the starchy kernels. The small difference in calculated niacin based on Tx61M and T13 could result from the use of a slight underestimate for the inherent concentration of Tx61M and/or an overestimate for T13. An overestimate for T13 might also result from a niacin-contributing power for T13 somewhat below its assay value. It should be noted, however, that such difference, if any, is less than in the T13 crosses with Tx61M.

The average of 21.7 for the estimate of contributing power (table VIII) based on backcrosses and that of 21.4 for the estimate based on selfs of the heterozygotes are in satisfactory agreement. It is of some interest, however, to check such agreement in another way. Using the values of 21.7 for the average of the sweet parents, 17.9 for Tx61M, and 51.2 for T13, the fol-

lowing can be obtained: for the selfs of (Tx61M × *su*), a calculated concentration of 19.8 to compare with an observed 20.3; and, for the selfs of (T13 × *su*), a calculated concentration of 36.5 to compare with an observed 37.9. Insofar as the averages are concerned, then, there appears to be excellent agreement, indicating generally independent inheritance of the *su* gene and the multiple factors coming in from the two parents.

For a further check on this situation, additional seed of the F_1 crosses was planted in 1949 and selfed ears obtained. The niacin concentrations of the starchy and sugary kernels are shown in table IX, the values for 1948

TABLE IX

NIACIN CONCENTRATION IN STARCHY (*Su*) AND SWEET (*su*) KERNELS FROM SELFED CROSSES OF Tx61M AND T13 STARCHY INBREDS WITH FIVE SWEET INBREDS, AS GROWN IN 1948 AND IN 1949, AND THE EXCESS CONCENTRATION OF THE *su* KERNELS IN MICROGRAMS/GRAM AND AS A PERCENTAGE OF THE STARCHY CONCENTRATION.

Sweet parent and year		Starchy parent Tx61M				Starchy parent T13			
		Kernels		Difference		Kernels		Difference	
		Su	*su*			*Su*	*su*		
		Micrograms/gram			%	Micrograms/gram			%
P14	1948	20.0	28.7	8.7	44	31.1	49.3	18.2	59
	1949	19.7	29.0	9.3	47	37.2	59.1	21.9	59
P39	1948	17.8	25.5	7.7	43	34.5	58.0	23.5	68
	1949	15.3	22.3	7.0	46	28.1	54.0	15.9	57
39K2	1948	....	....	....	....	44.6	65.3	20.7	46
	1949	19.2	28.8	9.6	50	33.4	48.9	15.5	46
51K2	1948	23.0	37.8	14.8	64	41.5	85.1	43.6	105
	1949	17.6	29.9	12.3	70	36.9	66.9	30.0	81
145	1948	....	....	....	....	34.6	54.9	20.3	59
	1949	13.7	21.0	7.3	53	29.5	42.5	13.0	44
Mean*	1948	20.3	30.7	10.4	51	37.3	62.5	25.2	68
	1949	17.5	27.1	9.6	55	33.0	54.3	21.3	65
S.E.**	1949	1.2	1.7	2.1		2.3	3.9	4.5	

*Means based on progenies represented in both years.
**Generalized. Per pedigree or difference. Standard errors for 1948 shown in table VII when available.

being shown with them for comparison. The estimates based on the 1948 data of 19.8 and 36.5 agree satisfactorily with the observed 1949 concentrations of 17.5 and 33.0 for the *Su* seeds from the Tx61M and T13 crosses. The slightly lower concentrations in 1949 may well have been due to the change in assay methods described earlier in this paper.

It was noted in connection with table VII that the excess concentration for the sweet kernels over that of their starchy sibs was not uniform, but differed both with the individual sweet parents and with Tx61M and T13. These excesses for the selfed progenies of 1948 and 1949 have been computed

in percentages of the starchy sibs and are shown in table IX. The average for all progenies is 60%. The average excess for all *su* seeds in the backcross progenies shown in table VII is 60.5%. It would seem, then, that this might well be taken as the average extent to which the *su* gene increases the niacin concentration of average starchy complexes. But, as in many cases, averages do not mean everything. Thus, the *su* gene coming in with 51K2 is more potent in increasing niacin concentration than the *su* coming in from the other sweet inbreds. A gene closely linked with *su* could, of course, produce a similar effect. Our data are inadequate to show whether the *su* coming in from 51K2 differs genetically from that (or those) coming in from the other sweet inbreds. The data are adequate, however, to suggest such a possibility. And why should there not be several *su* alleles indistinguishable in their influence on the starchy-sugary situation, but differing in their influence on niacin concentration? Again, the *su* gene exerts a larger influ-

TABLE X

THE TOTAL AND FRACTIONAL VARIANCES IN NIACIN CONCENTRATION OF STARCHY (*Su*) AND SWEET (*su*) KERNELS FROM SEGREGATING SELFED EARS OF THE 1949 CROP, AND THE COEFFICIENTS OF CORRELATION FOR THESE VARIANCES AT DIFFERENT LEVELS OF ANALYSIS.

Source of variance	D/F*	Variance in		Correlation coefficient	
		Su	*su*	r	r^2
Total	29	82.1	266.0	0.95**	0.90
Between *Su*	1	1902.4	5916.5	1.00	1.00
Within *Su*	28	17.1	64.2	.77**	.59
Between *su*	4	61.5	263.5	.77	.59
Su × *su*	4	10.1	51.8	.61	.37
Within progeny	20	9.6	26.9	.84**	.71

*For variances, 1 less for correlations.
**Significant at 1%; others not significant.

ence, both absolute and relative, on a background of high-niacin germ plasm (T13 crosses) than of low-niacin germ plasm (Tx61M crosses).

The relation between the niacin concentration in comparable sweet and starchy material is best shown by the correlation for the starchy and sweet kernels coming from the same selfed ears of the 1949 crop. This is reported in table X, both for the total variance and for the portions of the variance coming in from the various sources. The material comprised 30 ears, three for the crosses of Tx61M and of T13 with each of the five sweet inbreds listed in table IX. The over-all correlation of 0.95 is highly significant and indicates that 90%—*i.e.*, r^2, of the variance was concomitant. The correlations of the fractional variances show a similar situation at all levels of analysis. Even though some of the coefficients of correlation are not significant by conventional statistical standards, their consistency indicates this to be the fault of too few degrees of freedom rather than inadequate correlation.

High-niacin sweet corn

The ease with which niacin concentration was increased in starchy corn (**12**) made it desirable to see what could be done in this direction with sweet corn. Moreover, an increase of niacin in sweet corn would add directly to the value of this vegetable for human food. Accordingly, some very limited plantings were made in 1949 from high-niacin sugary segregates from the T13-sugary crosses, and selfs and crosses were obtained in this material. The data in table XI show the niacin concentration of the parent ear planted in 1949 and of the progeny ears chosen for planting in 1950 for continued selection. While some of the results are disappointing, others are promising, and the final answer remains to be determined. To further the solution to this question, ears with lower as well as higher concentrations were included in the 1950 plantings.

TABLE XI

THE NIACIN CONCENTRATION OF THE SUGARY SEEDS SEGREGATING FROM SELFED EARS OF T13 CROSSED WITH EACH OF THREE SWEET CORN INBREDS AS PLANTED IN 1949, AND OF EARS OBTAINED IN THAT YEAR AND CHOSEN FOR PLANTING FOR CONTINUED SELECTION IN 1950.

1950 Pedigree	Niacin concentration micrograms/gram	
	Parent	Progeny
(T13 × P14)-1S-1	52.9	38.2
(T13 × P39)-1S-1	65.4	42.2
-4		74.0
-5		51.6
(T13 × 39K2)-1S-2X	66.2	60.3*
(T13 × 51K2)-1S-1	85.1	66.6
-2		91.8

*An ear crossed with (T13 × 51K2)-1S-1, all others selfed.

Discussion

Our earlier paper (**12**) was concerned with the inheritance of niacin concentration in starchy corn. We concluded that this depended on a multiple-factor system involving many genes of small individual effects. It was noted also that data from experiments by others (**9**) with starchy-sugary hybrids indicated simple Mendelian inheritance, with *Su* dominant for low niacin over its *su* allele. We suggested that this might be a pleiotropic effect of the *Su su* alleles. The data presented here support the conclusion as to multiple-factor inheritance in starchy corn, though suggesting some additional details. The present data also support our suggestion of a pleiotropic effect of the *Su su* alleles, and show further that these genes interact with the multiple-factor system in final determination of niacin concentration.

It seems proven that the niacin concentration of the corn endosperm (and, therefore, the kernel) is controlled almost exclusively by the direct

action of genes during its formation. Environment has been shown (**12**) to have a negligible influence. Even large differences in seed size, as here reported, had no important effect on niacin. The physiology of the seed-parent plant as influenced by the genes *Su* and *su* made no discernible difference. Finally, the evidence of a 2:1 ratio for the effect of seed and pollen parents appears conclusive. Our data, accordingly, are for the means of the designated populations, those for *De* and *de* and for *Su* and *su* phenotypes being, of course, distinctive for these characteristics.

Data on the effects of selecting further for high and for low niacin concentration in the Huffman variety will be published later. Continued gradual progress has been made, with extremes for individual seed ears of about 70 and 7 micrograms per gram having been obtained. This progress has been such as would be expected under multiple-factor inheritance. On the other hand, most of the crosses that have been made between high-niacin lines have had lower concentrations of niacin than the mean of the parents. In one series of crosses, moreover, the niacin was lowered less in crosses between more closely related material and lowered more in crosses of less closely related material. Such a situation would result if selection for high niacin had isolated strains carrying an accumulation of recessive, or partly recessive, genes for high niacin. Different strains would carry different assortments, and hybrids between strains would tend to have lower concentrations than the parental values would indicate. The behavior of T13 in its crosses, particularly with Tx61M and the sweet inbreds, also would be in keeping with such a hypothesis. Our crosses were made incidentally to the selection program and the data are indicative only on the above situation. It is an interesting question, however, and experiments are under way which should be more conclusive. The system would be one of multiple factors in either event, the difference being whether the determining genes lack dominance or whether dominant and recessive genes for plus and for minus niacin also are concerned.

The correlations for niacin concentration in the sweet and starchy kernels of individual ears and pedigrees is abundant evidence of the interdependence of the multiple-factor and *Su su* systems of inheritance in determining final niacin. On the other hand, the interaction was not strictly additive, as the concentration of the sweet kernels was larger both absolutely and on a percentage basis when the multiple-factor background was higher (T13 crosses) than when it was lower (Tx61M crosses). Finally, the *su* gene coming in from 51K2 was more effective in increasing niacin than the *su* genes coming in from the other sweet inbreds.

Any consideration of how the multiple-factor system acts in determining endosperm niacin concentration must be highly speculative at this time. However, some basic facts are available. Data will be published elsewhere to show that the so-called "niacin" of these studies is principally free nicotinic acid and not one of the coenzymes or nicotinamide as might have been expected. It is reasonable to assume that the genes which control the ulti-

mate level of nicotinic acid in starchy endosperm as well as the niacin-contributing power of sugary endosperm may be concerned not only with the biosynthesis of the vitamin but also with its metabolic degradation. Individually, these genes may complete new enzymatic pathways of synthesis and of degradation or replicate existing ones. Furthermore, they may complete sequential reaction series antecedent to nicotinic acid formation and thus contribute to lower niacin by introducing side-reactions involving precursors of the vitamin. The task of identifying niacin precursors in corn was begun in the Columbia laboratory by NASON (**11**). It is now clear that feeding tryptophane to excised embryos of T13, and feeding 3-hydroxyanthranilic acid to embryos of T13, P39, and their reciprocal crosses leads to appreciable accumulation of extra niacin. The situation so far resembles that in Neurospora as reviewed recently by BEADLE (**2**). There still remains the task of identifying other possible precursors, if any, and of comparing the relative rates of utilization of these precursors by genetic lines having intrinsically different niacin contents. A corresponding study of the breakdown of nicotinic acid to trigonelline is in progress. From this point of view, it would appear that the higher-niacin strains of corn might be those having a balance of plus niacin genes to promote synthesis, or a balance of minus niacin genes to retard degradation, or both.

It seems entirely possible that the influence of the *Su su* alleles on niacin concentration is associated more or less directly with their action on carbohydrate deposition in the endosperm. CAMERON and TEAS (**3**) speculate briefly on this possibility; and their data showing that *du* affects carbohydrate and niacin concentration similarly to *su* is excellent corroborative evidence. Our data can be explained under such a hypothesis. Thus, if niacin is used (degraded) during the conversion of soluble polysaccharides (or of some common intermediate) to starch, there would be less degradation in the sugary kernels in which there is less starch formed. Under such a hypothesis a higher-niacin sweet corn should have a lower starch content and consequently be sweeter and more tender. Thus, niacin assay might well be a useful tool in breeding sweet corn for improved quality as well as for better nutritive value.

Alternatively, our data can fit equally well the hypothesis that denies a direct relation between the two influences of the *Su su* alleles. Thus the *su* gene may simply be more efficient in fostering the synthesis of niacin from some precursor. Under either of these hypotheses there would be an increase in the niacin concentration of sugary seeds. In one case this would result from the absence of excessive degradation and in the other from the direct synthesis of a larger quantity of the vitamin.

It is difficult to believe, however, that a single gene would exercise separate (and presumably independent) control over two such widely related processes as nicotinic acid metabolism and polysaccharide production from the products of photosynthesis. It would seem much more likely that one process is closely linked to the action of the *su* gene and that the other represents an ensuing metabolic consequence. The presence of large amounts

of soluble sugars and water-soluble polysaccharides in corn endosperm during the development of the kernel is associated with the occurrence of correspondingly high concentrations of nicotinic acid and of other B vitamins. Presumably metabolic activity is high in the presence of these large quantities of respirable substrates. During the extensive starch grain formation that accompanies maturation of starchy kernels, the niacin concentration rapidly decreases, although the absolute amount per kernel continues to increase slowly. In the sugary kernel, however, less starch grain formation occurs, and the concentration of soluble carbohydrate remains high, as do also the concentrations of niacin and other water-soluble vitamins (**3**). In the physiological sense, therefore, the high niacin content of sugary endosperm may represent simply a case in which the demand for niacin in dehydrogenase formation is maintained at levels somewhat more characteristic of vegetative than of storage tissue. In this way there may exist an instructive parallelism between the composition of sugary endosperm and callous tissue induced by stem girdling when these are compared respectively with starchy endosperm and normal vegetative stem tissues.

Stocks developed in our genetic studies can prove invaluable in the collateral physiologic research necessary to the final solutions. Thus, the starchy strains developed differ among each other by different degrees of inbreeding, and therefore by graduated assortments of differentiating genes. The same will be true later for sweet strains. Both of these should lend themselves well to studies of physiologic processes differing in detail. The use of bulk samples of kernels will be unsatisfactory for such studies, but this should not be necessary. There seems to be no reason why assays should not be made of the endosperms of individual kernels, and the embryos of such kernels then be used for producing plants of the specifically determined phenotypes.

Summary

1. Niacin concentration in corn is a function of the genetic constitution of the endosperm, with practically no influence by kernel size, environment or by differences in the physiology of the seed-parent plants as between *Su su* and *su su* plants.

2. Within starchy corn, niacin concentration is inherited as a quantitative, multiple-factor character. Crosses are basically intermediate between the parents with the seed parent exercising twice the influence of the pollen parent.

3. There was evidence that some starchy lines are high in niacin because they carry one or more recessive high-niacin genes. Crossing such lines frequently resulted in niacin concentrations below those expected from the parental values.

4. The *su* gene was simply recessive for high niacin to its *Su* allele, the niacin concentration of sugary kernels averaging about 60% larger than that of the starchy kernels from the same progeny.

5. The niacin concentration of sweet corns is a poor criterion of how

much they will transmit to crosses with starchy corn, but this "inherent" niacin concentration of sweet corn can be estimated from crosses with starchy corn of known niacin concentration.

6. The *Su su* locus interacted with the multiple-factor system of inheritance in determining final niacin concentration. Thus, sugary segregates from crosses of sweet corn with high-niacin starchy corn had higher concentrations than corresponding crosses with low-niacin starchy corn, the over-all correlation for niacin in sweet and starchy kernels from segregating ears being 0.95 for 28 degrees of freedom.

7. The increase in niacin in the sugary kernels was not strictly additive, that in the higher-niacin strains being larger than that in the lower-niacin strains, both absolutely and relatively.

8. The *su* genes from different sweet inbreds differed in the degree to which they increased the niacin concentration above that for their *Su* alleles, suggesting *su* alleles differing in their niacin influence.

9. Selection for higher niacin among sugary segregates was successful in initiating strains of sweet corn with concentrations materially above those in the inbreds used at the beginning of these experiments, one selected ear from the 1949 crop having a concentration of 91.8 micrograms per gram.

10. Some of the implications of the results are discussed and possibilities for genetic-physiologic research with the stocks developed are suggested.

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TRYPTOPHAN, NIACIN, AND INDOLEACETIC ACID IN SEVERAL ENDOSPERM MUTANTS AND STANDARD LINES OF MAIZE[1]

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Kernels of sugary (su_1) maize have been shown to be higher in niacin than normal ones (**3, 4**) as have endosperms of waxy (wx) (**10**), amylaceous (su_1^{am}), and dull (du) (**5**). Sugary maize has also been found to be higher in total indoleacetic acid (**2**). There are reports of a tryptophan-niacin relationship in maize (**13**), tryptophan conversion to indoleacetic acid (auxin) in other plants (**17, 18**) and niacin-indoleacetic acid interaction in plant growth (**6, 7**). These findings linking the metabolism of tryptophan, niacin, and indoleacetic acid in plant tissues suggested the desirability of including assays for all three in a survey of endosperm biochemistry. The present investigation reports tryptophan, niacin, and indoleacetic acid levels in seven endosperm mutants and eight inbred lines of maize.

Materials and methods

The mutant types used are readily classifiable recessive characters: waxy (wx) kernels almost normal in size, opaque dull in appearance, starch stains red with iodine instead of blue; sugary-1 (su_1) (hereafter listed as *su* since no other sugary gene was included in this study) kernels translucent and wrinkled; shrunken-1 (sh_1) and shrunken-2 (sh_2) kernels opaque, smoothly indented or shrunken; brittle-1 (bt_1) and brittle-2 (bt_2) kernels slightly translucent, more shrunken than *su;* intermediate in appearance between *su* and *sh;* miniature seed (mn) kernel only about one-fifth normal weight, has papery cover over seed. The morphological study of LOWE and NELSON (**11**) indicates that *mn* seeds develop only to about 9 to 14 days after pollination.

A study of mutant and normal kernels for any endosperm type must involve consideration of the genetic background and constitution of kernels being compared as well as any differences in weight per kernel. One could compare a series of mutant lines and a series of normal lines; however, it would be difficult to decide which of normal lines having widely different tryptophan, niacin, and indoleacetic acid levels should be used for comparison. A more desirable alternative is the use of mutant strains that have been repeatedly crossed to and reisolated from a standard line. As the conversion is carried through more backcrosses the ideal is approached where the mutant and the standard line differ only by the mutant gene being

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studied. Converted lines were not available for most mutant genes. Another possibility, and that adopted in this study, is the comparison of mutant and normal kernels on the same ear. Both types of kernels are thus exposed to identical nutrition and environment during development. However, on an ear from a selfed heterozygous plant, the normal kernels are a mixture of genotypes. Since maize endosperm is triploid, if *m* represents a mutant gene, and + represents its normal allele, the genotypes of the four classes of kernels expected will be: *mmm*, *mm+*, *m++*, and *+++* (in equal numbers). Only the first type is a phenotypic mutant, the others are all normal. Inasmuch as the mutant genes in this study are expressed morphologically only when homozygous, it is reasonable to lump the last three classes as normals. If a mutant were an incomplete recessive then the *mm+* and *m++* types should be intermediate in chemical composition between *mmm* and *+++*. This should reduce the amount of difference between the mutant and normal, so that differences found in such comparisons would not be maximal.

Except for shrunken-2, brittle-2, and miniature seed, the examples selected for assay of each mutant represent different genetic backgrounds, thus tending to reduce the probability that linked genes are responsible for observed differences. In the case where a mutant has been crossed at least twice to an inbred line, the inbred is listed as background in table I.

Only degermed mature kernels were assayed. Embryos were removed from the air-dried kernels with a scalpel and the endosperms ground to 40 mesh in a Wiley mill. Niacin assays were carried out by the procedure of the ASSOCIATION OF VITAMIN CHEMISTS (**1**); tryptophan was determined by the method of STOKES *et al.* (**15**) using *Streptococcus faecalis* following pepsin and trypsin hydrolysis of samples according to WOOLEY and SEBRELL (**19**); total indoleacetic acid was measured colorimetrically by the method of TANG and BONNER (**16**) after release of precursor or bound auxin by hydrolysis at 100° C in pH 9.6 borate buffer and extraction with ether. Assay values for tryptophan and niacin represent the average of at least three levels of supplement, the indoleacetic acid values are averages of at least two and usually three separate determinations. Too few samples of the mutant material with different genetic background were available to warrant extensive analysis and statistical treatment. Further sampling of sister ears would have been of little value in establishing how general is the effect of a given endosperm gene on the chemical composition. Assay values are tabulated as micrograms per gram (γ/gm.) and micrograms per kernel (γ/K).

Results and discussion

From the data presented in tables I and II some generalizations can be made. Differences were considered significant if present in all three samples of a given genotype and they average more than 1.5 times as much as normals on a kernel basis. In the case of *mn*, where all values were less than

TABLE I

TRYPTOPHAN, NIACIN, AND INDOLEACETIC ACID LEVELS OF ENDOSPERM MUTANTS.

Strain	Background	Relative endosperm weight	Tryptophan		Niacin		Indoleacetic acid	
			γ/GM	γ/K	γ/GM	γ/K	γ/GM	γ/K
waxy (*wx*)	CC5	.96	181	17.9	53.6	5.3	8.8	.9
normal*		1.00	148	15.3	39.3	4.1	7.3	.8
waxy	WF9	.94	256	28.9	32.0	3.6	81.1	11.7
normal		1.00	376	57.6	24.9	3.8	64.3	9.8
waxy	R4	.98	334	51.4	21.4	3.3	21.5	3.3
normal		1.00	280	44.0	19.2	3.0	17.7	2.8
sugary-1 (su_1)	CC5	.83	526	84.7	53.2	8.6	29.0	4.7
normal		1.00	505	98.0	28.2	5.5	15.0	2.9
sugary-1	L317	.73	621	111	35.8	6.4	21.0	3.7
normal		1.00	415	102	16.9	4.1	11.9	2.9
sugary-1	KYS	.68	421	56.8	67.3	9.1	75.5	10.2
normal		1.00	265	52.2	22.5	4.4	54.0	10.6
brittle-1 (bt_1)		.46	786	111	120	16.9	50.4	7.1
normal		1.00	442	136	21.3	6.5	10.4	3.2
brittle-1		.51	846	110	97.0	12.6	46.9	6.1
normal		1.00	466	118	17.6	4.5	19.3	4.9
brittle-1		.42	1032	58.8	107	6.1	86.8	5.0
normal		1.00	550	74.3	24.3	3.3	41.6	5.6
brittle-2 (bt_2)		.68	921	97.6	77.7	8.2	92.0	9.8
normal		1.00	349	54.8	34.1	5.4	40.0	6.3
brittle-2		.71	906	140	59.4	9.2	74.3	11.5
normal		1.00	322	70.2	31.2	6.8	37.0	8.1
brittle-2		.77	971	111	57.1	6.5	84.0	9.6
normal		1.00	301	44.5	30.1	4.5	35.5	5.3
shrunken-1 (sh_1)		.87	386	72.6	58.0	10.9	15.3	2.9
normal		1.00	421	91.4	33.7	7.3	11.3	2.5
shrunken-1		.80	328	57.4	43.4	7.6	48.8	8.5
normal		1.00	145	31.9	28.4	6.3	28.3	6.2
shrunken-1		.84	511	111	52.2	11.4	25.7	5.6
normal		1.00	313	80.8	33.8	8.7	17.7	4.6
shrunken-2 (sh_2)		.48	982	75.6	116	9.0	36.3	2.8
normal		1.00	181	29.0	32.8	5.9	15.5	2.5
shrunken-2		.51	1024	112	85.4	9.3	38.4	4.2
normal		1.00	625	134	25.0	5.4	13.6	2.9
shrunken-2		.50	1126	115	72.7	7.4	31.8	3.2
normal		1.00	373	75.3	21.2	4.3	21.3	4.3
miniature (*mn*)		.23	382	27.2	28.0	2.0	9.7	.7
normal		1.00	238	72.6	25.0	7.6	17.9	5.5
miniature		.17	475	23.8	41.7	2.1	13.2	.7
normal		1.00	104	30.1	19.9	5.8	21.8	6.4
miniature		.29	424	26.3	36.8	2.3	14.7	.9
normal		1.00	187	40.6	20.0	4.3	19.0	4.1

* Normal kernels are from the ear on which previous mutant is segregating.

normal on a per kernel basis, a value of less than .67 times that of normal was considered significant. Thus: *wx* is not different from normal. LENG *et al.* (**10**) have recently reported a somewhat higher niacin level; *su* has higher niacin content, as previously found (**3, 4**). The amount of total indoleacetic acid on a weight basis is increased, as reported by AVERY *et al.* (**2**); bt_1 has increased niacin, but not indoleacetic acid. On a dry weight basis indoleacetic acid was 2.6 times normal, a finding in contrast to the results of AVERY *et al.* (**2**) who reported a *bt* sample to be normal in total indoleacetic acid; bt_2 has increased tryptophan and indoleacetic acid; sh_1 is not different from normal; sh_2 has increased niacin; *mn* has decreased tryptophan, niacin, and indoleacetic acid.

The differences that meet the mentioned criteria are approximately equal to or greater than those reported in literature for increased niacin content of *su*.

TABLE II

AVERAGE TRYPTOPHAN, NIACIN, AND INDOLEACETIC ACID RATIOS FOR MUTANT AND NORMAL ENDOSPERMS.

Mutant	Average ratio of mutant to normal					
	Tryptophan		Niacin		Indoleacetic acid	
	Per GM.	Per K.	Per GM.	Per K.	Per GM.	Per K.
wx	.96	.84	1.28	1.14	1.24	1.19
su	1.33	1.00	2.33	1.70	1.55	1.13
bt_1	1.83	.85	5.12	2.41	2.58	1.33
bt_2	2.89	2.06	2.03	1.43	2.25	1.56
sh_1	1.39	1.19	1.60	1.35	1.57	1.30
sh_2	2.66	1.27	3.48	1.65	2.11	1.06
mn	2.43	.54	1.65	.36	.64	.15

In the case of the seven endosperm mutants studied, as well as others reported for maize, the seed is reduced in size (**11, 12**). Therefore, comparisons of amount of a substance per kernel and amount per gram cannot show the same ratios for mutant/normal. The kernel as a biological unit has been given preference in interpretations, since the amount of substance per gram might show differences that are only a reflection of variation in amount of reserve carbohydrate storage per kernel. Two simple cases of effects due solely to amount of stored carbohydrate or kernel weight differences are as follows: (a) Those in which the amount of a substance per kernel for normal and mutant are the same. Since the weight of the mutant endosperm is reduced, the amount of the substance per gram is increased according to the ratio of kernel weights. (b) Those in which the amount of a substance per gram for normal and mutant are the same. Because of the reduced size of the mutant endosperm the amount per kernel is decreased.

If the mutant differs in the content of tryptophan, niacin, or indoleacetic acid in a way described by either (a) or (b) it is assumed that genetic conditioning of the amount of carbohydrate storage, or the endosperm size,

respectively, is the factor operative. As noted above, the content of at least one of the three substances in the mutant shows significant differences from normal on a kernel basis for all except sh_1 and wx, which meet the (a) criterion for simple differences. The other differences observed must be due to variations in composition of substance stored.

It is interesting to consider MANGELSDORF's suggestion (**12**) that endosperm mutants correspond to incompletely developed normals. HANSEN *et al.* (**9**) found that the percentage of zein and the proportion of carbohydrate present as sugars in immature normal kernels corresponded approximately to values for mature *su* and *bt*.

If a given substance has a great range of values during development, it should constitute a sensitive test of the arrested development hypothesis. Thus, indoleacetic acid would be the most likely to show differences between theoretical and observed values, niacin least likely, since they respectively show the most and least difference during development (unpublished data). In order to make comparisons for this study, a curve of dry weight per kernel was plotted against days from pollination for: a cross of two inbred lines, CC5 × L317; and R4 line; and two from literature, a Dent and a Pop corn of GROSZMANN and SPRAGUE (**8**). All four curves of dry weight per kernel were similar in shape. The first two, for which tryptophan, niacin, and indoleacetic acid levels were available (unpublished data) represented the most spread among the four. Assuming that the average percentage of normal development that the mutant attains on segregating ears is a valid index of the developmental stage, the time in days from pollination that showed the corresponding percentage of mature dry weight was read from the two curves. Tryptophan, niacin, and indoleacetic acid levels for the corresponding stages were taken from ontogenetic curves. The mutant and normal were compared as follows:

$$\text{for normal} \quad \frac{\gamma \text{ of substance per kernel for the developmental stage corresponding to weight of mutant}}{\gamma \text{ per kernel at maturity}} \quad \text{(calculated ratio)}$$

$$\text{for mutant} \quad \frac{\gamma \text{ per mutant kernel}}{\gamma \text{ per normal kernel}} \quad \text{(observed ratio)}$$

Although such comparisons should ideally be made only with the normal that represents the genetic background of each mutant, the use of ratios for comparison reduces effects of difference in genetic background. The range of calculated values for the two lines are given for comparison with observed ratios in table III. The largest differences between calculated and observed were found for indoleacetic acid. Waxy which is nearest to maturity on a kernel basis (.96) and shrunken-1 next nearest maturity (.84) showed relatively less difference between calculated and observed values. Since it is improbable that the amylose-amylopectin in *wx* duplicates any immature normal amylose-amylopectin ratio, only sh_1 can be explained as simple arrested development.

TABLE III

COMPARISON OF TRYPTOPHAN, NIACIN, AND INDOLEACETIC ACID LEVELS CALCULATED ON THE ARRESTED DEVELOPMENT HYPOTHESIS WITH OBSERVED VALUES.

Mutant	Equivalent age Days*	Tryptophan		Niacin		Indoleacetic acid	
		Calc.	Obs.	Calc.	Obs.	Calc.	Obs.
wx	33-42	.9-1.1	.8	1 -1.3	1.1	1.6- 7.1	1.2
su	26-33	1 -1.1	1.0	.8-1.5	1.7	3.2- 8.2	1.1
bt_1	21-24	.8-1.1	.9	.9-1.2	2.4	5.8-17.5	1.3
bt_2	26-32	1 -1.1	2.0	.8-1.5	1.4	3.4- 8.2	1.6
sh_1	28-37	.9-1.2	1.2	.9-1.6	1.4	2 - 9	1.3
sh_2	21-25	.8-1.1	1.3	.9-1.2	1.7	5.8-15	1.1
mn	17-19	.5- .9	.5	.6- .8	.4	2.9- 7.6	.2

* Forty-four days taken as maturity.

The wide range of assay values for standard inbred lines (table IV) illustrates the hazard involved in selection of a single or only a few normal lines. For example, comparison of the niacin level in a *su* KYS (high) with an L317 normal (low) or indoleacetic acid levels for a *wx* WF9 (high) with an N6 or CC5 normal (low) might lead to rather dubious conclusions. It is possible that the brittle sample assayed for indoleacetic acid by AVERY *et al.* (2) failed to show higher than normal values because of being present on a genetic background intrinsically low in indoleacetic acid. It is important, therefore, that such comparisons be limited to cases where genetic background is unlikely to obscure results. Such limiting could be accomplished for instance by using a large number of mutant and normal lines, by several examples of inbred line conversions, or by several examples of segregating ears if there is no effect of heterozygosity.

The values of 120 and 116 micrograms of niacin per gram for brittle-1 and shrunken-2 samples appear to be the highest reported for maize. The average of the series of eight standard inbred lines was 25.0 micrograms of niacin per gram including the high niacin T13.

TABLE IV

TRYPTOPHAN, NIACIN, AND INDOLEACETIC ACID LEVELS IN STANDARD INBRED LINES.

Inbred line	Tryptophan		Niacin		Indoleacetic acid	
	γ/GM	γ/K	γ/GM	γ/K	γ/GM	γ/K
KYS	491	80.0	33.6	5.5	53.5	8.7
L317	175	27.8	11.2	1.8	8.0	1.3
CC5	190	30.4	33.9	5.4	5.5	.9
WF9	301	37.5	24.8	3.1	67.9	8.5
R4	424	51.7	15.7	1.9	20.5	2.5
N6	67	12.6	8.1	1.5	3.7	.7
I205	430	98.9	22.6	5.2	27.3	6.2
T13	208	52.6	50.4	12.8	13.8	3.5

Germination of mutant types is often poor compared with standard lines. Also, some of the mutants associated with higher sugar content are especially susceptible to mold damage under field conditions. The possibility of economic advantage being taken of the mutants used in this study (other than sugary) because of higher tryptophan or niacin content is therefore remote. However, niacin or tryptophan levels as high or higher than any of the endosperm mutants assayed might be found due to mutations that have no effect on kernel morphology.

Summary

The tryptophan, niacin, and indoleacetic acid contents of mature maize endosperms of the genotypes waxy, sugary-1, brittle-1, brittle-2, shrunken-1, shrunken-2, and miniature seed from ears segregating for each were compared with values for normal kernels from the same ears. On a weight basis brittle-2, shrunken-2, and miniature had tryptophan contents more than 2 times normal; sugary, brittle-1, brittle-2, shrunken-1, shrunken-2 and miniature had 1.6–5.1 times normal niacin content; and sugary, brittle-1, brittle-2, shrunken-1, and shrunken-2 had 1.6–2.6 times the total indoleacetic acid content of normal. On a kernel basis some of the differences disappeared, inasmuch as mutant kernels are characteristically smaller than normals. Except for shrunken-1 the difference in assay values for mutant and normal cannot be explained as due to simple arrested development, differences in size of kernels, or to storage of a given amount of these substances per kernel irrespective of size.

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MORPHOLOGICAL AND PHYSIOLOGICAL RESPONSES OF CARNATION AND TOMATO TO ORGANIC PHOSPHORUS INSECTICIDES AND INORGANIC SOIL PHOSPHORUS[1]

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(WITH FOUR FIGURES)

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During the last few years considerable attention has been focused upon the use of the organic phosphorus compounds hexaethyl tetraphosphate and tetraethyl pyrophosphate as the active components of certain commercial insecticides. These substances are known to be toxic not only to insects but to other organisms in general as well. As the names of hexaethyl tetraphosphate and tetraethyl pyrophosphate are generally abbreviated HETP and TEPP, respectively, they will be referred to as such in this paper. HALL and JACOBSEN (**11**) concluded that HETP does not possess a definite structure by demonstrating that it is essentially a mixture of esters containing ethyl metaphosphate, triethyl orthophosphate, and tetraethyl pyrophosphate. These workers believe that the biological potency of the so-called HETP is actually due to the TEPP that it contains, although in a later publication, HALL (**10**) implied that HETP contains other toxic principles in addition to TEPP.

These compounds become of interest to the plant scientist through the formative effects that they elicit in particular sensitive species and this paper reports some of the fundamental effects that these compounds, or their degradation products, have on plant metabolism. The formative effects produced in plants by these compounds so strikingly resemble, in most cases, those of the hormone-type phytocides that a number of workers have attributed the symptoms to 2,4-D contamination of either the insecticides or spray equipment. Carefully controlled experiments have demonstrated, however, that these characteristic symptoms can be initiated consistently by HETP and TEPP in carnation, tomato, and sunflower. This has been the case generally when slightly higher concentrations than recommended for insect control were employed. Other reports (**24, 26, 27, 34**) have appeared confirming this result for the same and other species. SMITH *et al.* (**26, 27**) have noted that tomatoes, roses, chrysanthemums and carnations are susceptible to HETP and TEPP and they cite many grower reports of formative effects appearing in greenhouse ornamentals treated with these compounds. ZIMMERMAN and HARTZELL (**34**) have noted that epinasty was produced in more than 20 species when exposed to HETP and TEPP. The report of MCILRATH (**24**), as well as many Texas grower reports of 2,4-D-

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like symptoms in their cotton fields following the use of organic phosphorus insecticide sprays, adds cotton to the list of susceptible species.

Although carnation was used in the early studies with HETP, tomato was employed in most of the experimental work presented in this paper because of its unusual sensitivity to both HETP and TEPP. The similarity between the formative effects produced by the organic phosphorus compounds and the 2,4-D-type hormone suggests a common mode of action in plant metabolism and indicates a possible explanation of the biological link interrelating physiological function of hormones to the morphological growth response that they effect.

Preliminary experiments with carnation

BACKGROUND OF THE PROBLEM.—The symptoms, subsequently found to be caused by HETP, were first observed in 1948 in a commercial greenhouse at Lexington, Kentucky.

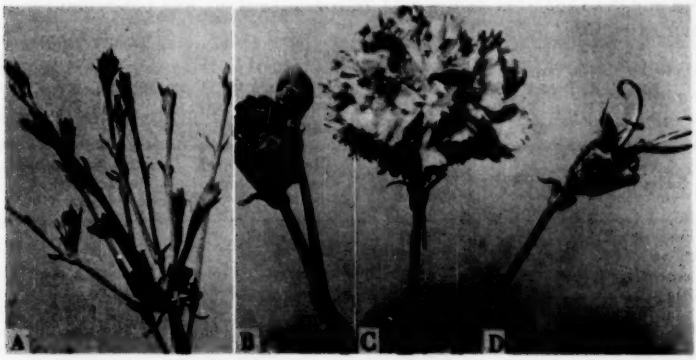


FIG. 1. Aberrated and normal carnation flowers. Left to right: A. Distorted, branched, flowering shoot. B. Normal bud. C. Normal flower. D. Distorted over-developed flower and lack of petals.

The abnormalities were confined to the flower or flowering shoot and consisted primarily of two general distortion patterns. In one case (fig. 1 A) the flowering stalk became repeatedly branched so that the flowers were borne in a cluster. Each flowering stalk developed numerous, as many as 10 to 15, small commercially inferior flowers. The other extreme (fig. 1 D) was characterized by having the stamens and pistil excessively over-developed and protruding from the calyx tube one and a half inches or more. The sepals were unusually large with a thick leathery appearance, whereas, the petals did not differentiate or in some cases appeared to have differentiated into stamens. The ovary was also prominent and contained mature seeds long before the vegetative parts of the flower had developed or emerged. The contrast with the arrangement and the developmental pattern of a

normal bud and flower (fig. 1 B, C) is brought out by the photograph and by reference to Bailey's treatment of the carnation (**2**).

Investigation revealed that the carnation benches had been fertilized yearly and received especially heavy applications of superphosphate. The grower also reported that the carnation houses had been sprayed twice with a commercial insecticide formulation containing HETP. The last spraying occurred about the time the floral buds were differentiating and appearing.

OBSERVATIONS AND RESULTS.—Several soil samples from each bench were collected, their pH measured electrometrically and the soluble mineral content determined colorimetrically or turbidimetrically for the elements calcium, phosphorus, nitrogen, and potassium by the methods of WOLF and ICHISAKA (**33**). Triplicate representative samples of shoots of normal and distorted plants were collected, their fresh and dry weights determined, and the dried material ground to pass an 80-mesh screen. Samples were then wet ashed with sulphuric acid–salicylic acid and 30% hydrogen peroxide and analyzed for total phosphorus, nitrogen, calcium, and potassium colorimetrically (**33**).

TABLE I

FRESH AND DRY WEIGHT OF CARNATION SHOOTS IN GRAMS, PER PLANT, PERCENTAGE DRY WEIGHT, TOTAL P, TOTAL N, TOTAL K, TOTAL Ca AS PERCENTAGE OF DRY WEIGHT PRODUCTION.

Sample fractions	Normal flowering control	Distorted flower, over-developed type	Distorted, branched, flowering-shoot type
Fresh weight	39.00	34.00	30.00
Dry weight	6.36	6.29	5.79
Percentage dry weight	16.30	18.50	19.30
Total P	0.31	0.46	0.35
Total N	3.01	1.55	1.42
Total K	2.73	4.23	2.26
Total Ca	0.40	0.56	0.41

In all cases the soil reaction of the benches was close to neutrality or slightly acid. Invariably the lower pH values of the soil were correlated with the highest amounts of soluble phosphorus and the frequency and degree of the flowering distortions (fig. 1). The amounts of soluble calcium, nitrogen, and potassium in the bench soil were found to conform to the recommended levels for optimum greenhouse culture of carnations.

It was noted (table I) that the distorted plants had a slightly higher total phosphorus content than the normal flowering plants. The concentration of calcium in the plants in general followed the phosphorus trend as shown by a gradual increase with increasing phosphorus. As has been noted previously (**4, 33**), the phosphorus and nitrogen fractions displayed a reciprocal relationship in that as phosphorus increased the total nitrogen decreased (table I). The level of potassium varied considerably among the three samples but tended to increase somewhat proportionately with phos-

phorus particularly in the high phosphorus plants (table I, fig. 1 D). The normal flowering controls produced a greater fresh and dry weight than did the distorted plants (table I). The percentage dry weight, as well as observation at the time of sampling, indicated that the abnormal plants were more woody than the normal flowering plants.

REPRODUCTION OF THE SYMPTOMS BY EXPERIMENTATION.—The preliminary observations and analyses (table I) indicated that the factors most likely causing the symptoms, either singly or collectively, were the high soil phosphorus or HETP spray. Therefore formal experiments were performed to determine the role of these factors in inducing the symptoms.

Carnation plants were propagated from cuttings taken from mature healthy plants grown in a commercial greenhouse at Lexington, Kentucky. When vigorously rooted, 20 plants each of the varieties Ovilette, Netta, and Northland were transplanted to 3-inch pots containing rich greenhouse soil. One month later the plants were transplanted to 8-inch pots, in which they were grown to maturity. At the second transplanting each variety was divided into four experimental lots of five plants each, making a total of 15 plants per treatment. Lot 1 was retained in ordinary greenhouse soil containing 175 p.p.m. of soluble P. Lot 2 was grown in soil composed of 10 parts of ordinary greenhouse soil (lot 1 soil) to 1 part of 20% superphosphate. Lot 3 received the same cultural treatment as lot 1 except they were sprayed with aqueous HETP at the preflowerbud stage. Lot 4 was identical to lot 2 except they also received the HETP spray treatment concurrently with lot 3. During and after the spray application lots 3 and 4 were segregated from lots 1 and 2 in the greenhouse. The HETP was applied to runoff with a hand atomizer sprayer at the rate of 600 p.p.m. in water. Two milliliters of Grasselli spreader was added to each liter of HETP solution to facilitate absorption. The plants were then left under normal greenhouse conditions and observed for a 2-months period. By that time neither the control plants nor the high phosphorus plants without spray (lots 1 and 2) had displayed any formative responses. Essentially the same distorted patterns shown in figure 1 were produced, however, by the HETP spray in lots 3 and 4, and the frequency and degree of the symptoms was approximately the same in either treatment. It is believed that the branched flowering-stalk pattern (fig. 1 A) was the result of excessive accumulation of HETP between the leaves and buds of the growing point, when applied at the early stages of flower development, and from which the abnormal growth developed. The overdeveloped floral pattern (fig. 1 D) no doubt resulted from HETP when applied to more mature flower buds later in their reproductive cycle.

Experiments with tomato

METHODS AND PROCEDURE

EXPERIMENTAL PLAN.—The investigation was designed to be conducted in four phases as follows: (1) To study and record the formative, structural

and compositional effects of TEPP on tomato over a fairly wide range of concentrations, applied both as an aqueous spray to the foliage and when added as a water solution to the soil (800 p.p.m.). (2) To determine the effects of high phosphorus soil fertilization on the growth and composition of tomato and to determine if extremely high soil applications of inorganic phosphorus would induce formative effects. (3) To determine the effect and possible interrelation of soil applications of inorganic phosphorus (phase 2) and TEPP on plant composition and respiration 48 hours after application. (4) To study and compare the active fractions in technical TEPP (40%) and relatively pure TEPP (98.1%) and to elucidate, if possible, the basic mechanism of their responses in plants.

Preliminary work disclosed that the range of 150 to 4,000 p.p.m. of TEPP covered all the practical degrees of response in tomato. The response produced was greatly modified, however, by the time of year. The above range of concentration was employed, using 40% technical TEPP, to achieve the objectives of phases 1, 2, and 3 of the study. The aqueous spray was applied with a hand atomizer sprayer until the foliage was wet. On the average wetting the foliage required about 5 ml. of solution for young plants and about 10 ml. for mature plants. Grasselli's spreader (tested and proven to be inactive in inducing the TEPP response) was added to the spray to facilitate absorption. In the fourth phase relatively pure TEPP at 400 p.p.m. was used, while in the respiratory measurements 400 and 800 p.p.m. concentrations were employed.

Culture of plants.—Certified seeds of tomato, *Lycopersicon esculentum* Mill, var. Marglobe, were germinated in trays of vermiculite under greenhouse conditions. When about two to three inches in height the seedlings were transplanted to 8-inch pots and grown to the desired experimental stage in a soil compost mixture of Houston black clay, Lufkin fine sandy loam, decomposed manure, and oat hulls. For the first phase of the work young plants were sprayed at the macroscopic flower bud stage. To minimize seasonal variations in response, the TEPP was applied during the months of December, February, and June and the data for this phase is the average total of 15 plants per treatment.

To determine the effects of extremely high levels of inorganic phosphorus nutrition, singly and in combination with spray applications of TEPP at 400 p.p.m., plants were cultured in a mixture of the above soil compost and 20% P_2O_5 in the ratios of 40:1, 20:1, and 10:1 (phases 2 and 3). As a matter of convenience these levels are designated as low phosphorus, medium phosphorus, and high phosphorus. Twelve plants were grown at each level of inorganic phosphorus, four from each level were sampled at the first flower cluster stage and four were harvested at the mature fruit stage. The remaining four plants in each treatment were employed for studying the combined effects of TEPP spray and inorganic phosphorus nutrition (phase 3) and were sprayed at the mature fruit stage and harvested 48 hours after spray application. Eight control plants were cultured in the soil compost

without added phosphorus, four being sampled at the first flower cluster stage and four at the mature fruit stage. The unsprayed plants of the three phosphorus levels at the second sampling served also as the controls for the 48-hour TEPP treatment of phase 3. Leaves used in the respiratory measurements were taken from healthy young vegetative plants grown in the compost mixture.

In the fourth phase, both young vegetative and mature plants grown in the soil compost were employed.

SAMPLING, ANALYTICAL, RESPIRATORY, AND ETHYLENE DETECTION METHODS.—Except for the experiment of the first phase to determine the effect on flowering (which ran for 90 days) and the 48-hour experiments the plants were sampled one month after TEPP application. Entire shoots of the plants of the first phase were harvested, all plants of the same treatment combined, and fresh weights determined. The composite samples were then dried in a forced draft oven at 80° C. The samples of the second and third phases were composed only of leaves. After drying to constant weight, dry weights were determined gravimetrically and all samples ground to pass an 80-mesh screen.

The analytical methods used for the labile carbohydrate determination are given in detail by EATON and RIGLER (**6**). Briefly, the sugars were extracted from the oven-dried samples in a Soxhlet apparatus with 80% ethanol and determined by the semi-micro method of WILDMAN and HANSEN (**32**). Sucrose was determined by hydrolyzing an aliquot of the extracted sample with concentrated HCl and computing in the usual manner. Starch contained in the Soxhlet residue (from the sugar extraction) was determined by combined diastatic and acid hydrolysis. The starch-free residue was hydrolyzed by autoclaving with HCl and the reducing values determined expressed as hydrolyzable residue.

Samples for total nitrogen, phosphorus, potassium, and calcium analyses were digested with combined sulphuric-salicylic acid and 30% hydrogen peroxide and their respective values determined colorimetrically (**33**) in a Fisher electrophotometer. Soluble phosphorus was extracted from oven-dried material with 70% ethanol and the extract analyzed colorimetrically for this fraction by the above method. Residual, or 70% alcohol-insoluble phosphorus, was calculated as the difference between total and soluble phosphorus. Determinations for potassium and calcium were checked in a Beckman D. U. Spectrophotometer and found to be in close agreement with the measurements made by the Fisher electrophotometer.

The respiratory rate of tomato leaves was measured, before and after spraying with TEPP, in a standard Warburg apparatus by the modified Warburg respirometer leaf-disc method reported by KLINKER (**18**). Oxygen uptake was measured with two-tenth ml. of 20% KOH in the center well to absorb carbon dioxide. Carbon dioxide was determined by the "direct" method of WARBURG (**29**). All measurements were made in a darkened room at 28° C and with a shaker rate of 120 strokes per minute. The equipment was allowed to equilibrate 15 minutes before starting the measure-

ments. The data presented are the averages of six replications all in close statistical agreement. The plants were sprayed with TEPP two hours prior to cutting the leaf discs used in the Warburg measurements.

To detect ethylene evolution the "triple response" of etiolated Alaska pea seedlings was employed. This variety is known to respond to as little as 0.05 p.p.m. of ethylene in air. The seed was germinated and cultured according to the technique of PRATT and BIALE (**25**). When about 4 cm. high, one Petri dish each of the seedlings was enclosed individually in sealed desiccators with young tomato plants previously sprayed with 400 and 800 p.p.m. TEPP. To test the possibility that TEPP gives rise to ethylene by degradation, a beaker containing 400 p.p.m. of TEPP in water was placed in another desiccator with the etiolated seedlings. In addition, controls of two types were used, unsprayed tomato plants with the etiolated seedlings and the etiolated seedlings alone.

To determine if the effects of adenosine triphosphate were comparable to TEPP, eight young pre-flowering tomato plants were sprayed to run-off with 250 p.p.m. of the Di Barium salt. Eight comparable plants received the same amount of ATP except by soil application and eight untreated plants served as controls.

Results, phase 1

GENERAL OBSERVATIONS

Plant symptoms and formative effects produced in tomato by TEPP (or its degradation products) can be roughly classified into three types: lethal, inhibitive, and stimulatory. In most cases, concentrations above 2000 p.p.m. were lethal; 800 to 2000 p.p.m. generally produced temporary inhibition, however, in time stimulation of growth resulted. This fact is in agreement with the known effects of growth regulators of the auxin type. The third type of almost immediate stimulation was produced by concentrations from 100 to 400 p.p.m. Further dilution was essentially without effect. The demarcation between these arbitrary types and TEPP concentration was not sharp, but they often merged into one another, and were subject to a factor which at present is called the "seasonal" effect (different responses produced by the same concentrations according to the season of the year applied). This seasonal effect has been noted by others (**24, 26, 27**). Immediate responses photographed five days after application are shown for the various concentrations (fig. 2).

Plants became severely wilted within two hours after application of solutions above 2000 p.p.m., the foliage dried to a gray-brown color and appeared dead within 24 hours after spraying. Some of the first recognizable symptoms were epinasty, almost immediate chlorosis of foliage (fig. 3 B), and wilting and drying of the growing point. The long term effects of TEPP on leaves, stems, and reproductive organs, which markedly resemble those induced by 2,4-D, are shown in figures 3 and 4. One difference, how-

ever, seems to be a greater effect of TEPP on the terminal meristem. In most cases, concentrations of 800 p.p.m. or above produced either death or complete inhibition of the terminal growing point. This resulted in the breaking of apical dominance and the forcing of the lateral branches (compare fig. 3 F and fig. 4 A, B, C). This TEPP response in tomato is somewhat similar to that reported for maleic hydrazide (9). In accord with

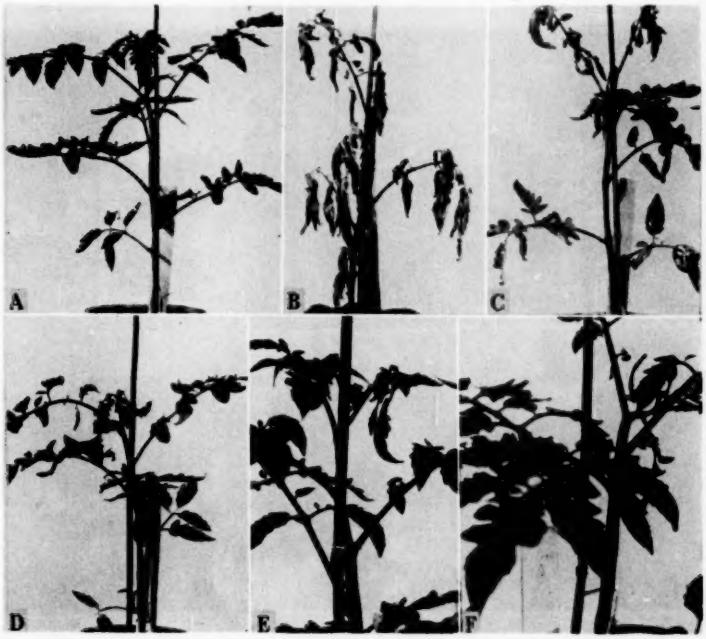


FIG. 2. Effects of TEPP at various concentrations on tomato five days after application. A. Control. B. 2000 p.p.m.—lethal dosage. C. 800 p.p.m.—inhibitive range (note effect on terminal meristem and young branches). D. 400 p.p.m.—stimulatory range. E. 300 p.p.m. (note effect on growing point). F. 200 p.p.m. (arrow points to twisted, distorted growing point).

SMITH *et al.* (26, 27), it was also noted that young plants were much more severely affected by TEPP than mature plants.

EFFECTS ON GROWTH AND METABOLISM

STEM ELONGATION.—Young tomato plants at the flower-bud stage of development were selected for uniformity of size and the stem heights measured just prior to spray application of TEPP and again one month later (table II). The data showed that 400 p.p.m. marked the dividing line between stimulation and inhibition of stem elongation.

FLOWERING.—An experiment extending over a period of 90 days was performed to check for stimulatory effects on flowering (table II). The results indicate that days to flowering was shortened by the concentrations at and below 400 p.p.m. SMITH *et al.* (**26, 27**) observed that roses showed increases as high as 30% in flower production following an aerosol HETP application in the summer months. The effects of TEPP on flowering apparently deserve more careful investigation and further work is contemplated.

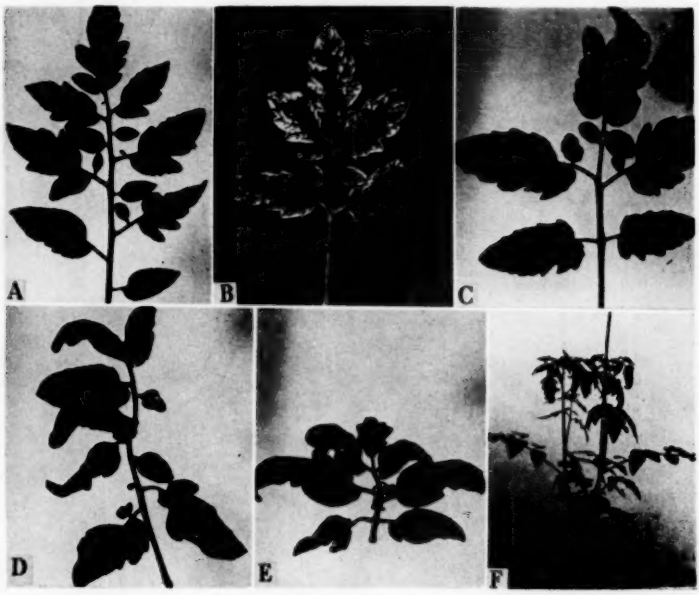


FIG. 3. Long term TEPP effects on foliage of tomato, morphological habit, and similarity of 2,4-D symptoms. A. Control leaf. B. Treated leaf showing mottled chlorosis. C. Slight formative effect appearing at 200 p.p.m. concentration. D. Increasing severity of foliage symptoms with increasing TEPP concentration (800 p.p.m.). E. Extreme leaf formative effect induced at 2000 p.p.m. F. Effect of TEPP on plant habit, resulting in breaking of apical dominance and forcing of laterals to produce twin-shoot type.

WEIGHT PRODUCTION.—The data (table II) show no evidence of increased dry weight from the application of TEPP. The general decrease in the dry weight of the sprayed plants suggests either accelerated plant catabolism or decreased anabolism by TEPP, at least over a 30-day period.

CARBOHYDRATE COMPOSITION.—Carbohydrate analyses (table III) disclosed that, in general, the treated plants at the higher concentrations decreased in carbohydrate content. The large decrease in sucrose and reducing sugars at 2000 p.p.m. suggests statistical significance. In most cases,

TEPP caused accelerated hydrolysis of reserve carbohydrates to the soluble forms and apparently increased respiration. For this reason, and because of the results of the growth measurements, the Warburg measurements were made. These are presented in a later section.

ELEMENTAL COMPOSITION.—Analyses showed that total phosphorus increased in sprayed plants somewhat in proportion to TEPP concentrations (table III). Additional fresh samples were washed in running tap water for

FIG. 4. Effects of TEPP and active fractions on vegetative and reproductive structures of tomato. A. Control plant. B. Branching induced by TEPP, arrow points to approximate height at time of application and slight swelling on the stem. C. TEPP-sprayed plant showing malformed, parthenocarpic fruit. D. Young plant self-rejuvenated from old plant killed by 4000 p.p.m. application and still manifesting slight formative effects. E. Mature plants sprayed with tetraethyl pyrophosphate (pure TEPP); diethyl hydrogen phosphate (DEHP) sprayed plant; and triethyl phosphate (TEP) treated plant. F. Young plant sprayed with DEHP and grown to maturity (note extreme 2,4-D-like symptoms).

three hours before oven drying but subsequent analyses revealed no difference between washed and unwashed samples indicating that there was no surface residue. The concentration of nitrogen increased in treated plants up to the 4000 p.p.m. level. Potassium increased only above 400 p.p.m. which was the limit of increased shoot height. Total calcium followed the phosphorus trend and increased throughout with increasing TEPP concentration. Except for converse results in the nitrogen trend the analyses of table III agree in general with those found for carnations (table I).

TABLE II

GROWTH AND FLOWERING RESPONSES OF GREENHOUSE, SOIL-GROWN TOMATO PLANTS OF PHASE 1. EFFECTS OF TEPP ON STEM ELONGATION AND WEIGHT PRODUCTION OF SHOOTS (30 DAYS AFTER APPLICATION) AND FLOWERING. EACH TREATMENT REPRESENTS THE AVERAGE OF 15 PLANTS.

TEPP treatment in p.p.m.	Stem elongation			Weight production			Flowering	
	Initial height	Final height	Change	Fresh wt. per plant	Dry wt. per plant	Per cent. dry wt.	Age at first flowers	Average fls. per plant to 90 days
	cm.	*cm.*	*cm.*	*gms.*	*gms.*	%	*days*	*no.*
Control	34	63	29	135.8	16.4	12.1	65	24
150	31	72	41	120.9	14.2	11.7	55	26
200	34	80	46	140.4	16.7	11.9	54	28
300	35	80	45	107.9	13.6	12.6	51	25
400	30	65	35	93.9	11.6	12.4	60	25
800	32	57	26	103.4	12.0	11.6	70	20
2000	31	50	19	54.3	6.4	11.8	82	12
4000	30	Lethal	Lethal	9.8	3.1	31.7	Lethal	Lethal
800 Soil	30	61	31	92.8	11.9	12.9	58	26

TABLE III

COMPOSITIONAL EFFECTS OF TEPP ON CARBOHYDRATE AND ELEMENTAL FRACTIONS OF GREENHOUSE SOIL GROWN TOMATO SHOOTS OF PHASE 1, 30 DAYS AFTER APPLICATION. EACH TREATMENT IS THE COMPOSITE OF 15 PLANTS.

TEPP treatment in p.p.m.	Carbohydrates					Elemental composition			
	Reducing sugars	Sucrose	Starch	Hydrolyzable residue	Total	Total N	Total P	Total K	Total Ca
Control	2.93	1.54	4.02	9.31	17.80	1.30	0.25	3.00	2.56
150	2.15	2.29	3.90	8.94	17.28	1.75	0.26	3.00	2.49
200	3.01	2.05	4.42	8.35	17.83	2.00	0.26	2.80	2.58
300	2.15	2.43	4.23	9.55	18.36	1.50	0.27	2.95	2.66
400	2.49	1.46	3.62	8.43	16.00	1.55	0.27	2.90	2.68
800	2.50	1.28	3.01	10.05	16.84	1.60	0.32	3.20	2.70
2000	0.09	0.04	2.91	9.03	12.07	1.65	0.38	3.50	2.77
4000*	2.58	0.02	6.46	8.56	17.62	0.95	0.40	3.40	2.56
800 Soil	2.45	1.86	4.03	9.51	17.85	1.40	0.25	2.85	2.50

* Lethal dosage but dead plants sampled.

Results, phase 2

General observations

Very little external differences were exhibited among the low 40:1, medium 20:1, and high 10:1 phosphorus plants, except perhaps for a slight increase in stem diameter with increasing phosphorus supply. All plants were extremely green and luxuriant and displayed no apparent phosphorus injury or formative effects. The controls were lighter green and much smaller in size than the low phosphate plants. The most abundant flowering occurred at the low (40:1) phosphorus level and all phosphate-treated plants flowered earlier than the controls.

Weight production.—The plants grown in the phosphorus-enriched soil produced considerably more fresh and dry matter than controls at both sampling dates (table IV). Dry weight increased proportionately with phosphorus fertilization at the first analysis, but the medium level plants had elaborated the most dry weight at the final sampling (table IV).

Carbohydrate composition.—At the first harvest, as phosphorus supply increased, all carbohydrate fractions decreased (table IV). The controls produced a greater total amount of carbohydrates than the high phosphorus plants, which were particularly low in reserve carbohydrates.

At the final harvest the medium 20:1 level plants elaborated significantly higher amounts of carbohydrates, but in the high phosphorus 10:1 plants, carbohydrates decreased sharply, although still above the controls. These trends in carbohydrate production are in general agreement with Eaton's work with sunflower and soybean (**7, 8**) but the levels of phosphorus employed in Eaton's work and the present study actually are too divergent for valid comparison of data.

Elemental composition.—Total phosphorus and nitrogen increased in plants somewhat proportionately with the level of phosphorus fertilization at the early analysis (table IV). Potassium, however, increased with increasing phosphorus application only up to the medium level. Calcium varied erratically and exhibited little correlation with any phosphorus levels. At the mature stage, analyses revealed that phosphorus still increased with added soil phosphorus but the percentage content of the other elements varied considerably, although the high phosphorus 10:1 plants in general contained the highest amounts (table IV).

Results, phase 3

Carbohydrate composition.—When TEPP was applied to the aerial organs of mature plants and the experiment terminated 48 hours after the spray treatment, the subsequent analysis (table V) gave support to the idea of accelerated hydrolysis and respiration suggested by the data of phase 1. At all levels the treated plants showed decreased carbohydrate reserves (hydrolysis) and increased soluble carbohydrates compared to untreated controls (table V). In addition to accelerating enzymatic hydrolysis the

TABLE IV

EFFECT OF INORGANIC PHOSPHORUS LEVEL ON WEIGHT PRODUCTION IN GRAMS, CARBOHYDRATES AND ELEMENTAL COMPOSITION AS PERCENTAGE OF DRY WEIGHT PRODUCTION OF TOMATO LEAVES OF PHASE 2 AT THE TWO DATES INDICATED. EACH TREATMENT REPRESENTS THE AVERAGE OF FOUR PLANTS.

Inorganic phosphorus level	Weight production		Carbohydrates					Elemental composition			
	Fresh wt. per plant	Dry wt. per plant	Reducing sugars	Sucrose	Starch	Hydrolyzable residue	Total	Total N	Total P	Total K	Total Ca
First sampling—first flower cluster stage											
Control	53.5	6.4	2.52	1.30	4.35	4.60	12.77	1.25	0.22	2.95	2.52
Low P	73.6	8.7	3.01	2.59	7.18	6.13	18.91	0.95	0.40	4.00	1.40
Medium P	94.4	11.3	2.93	2.09	5.67	5.47	16.16	2.10	0.42	4.50	1.95
High P	92.8	14.1	2.72	1.34	3.69	3.53	11.28	2.15	0.59	4.40	1.10
Second sampling—mature fruit stage											
Control	94.8	13.3	1.53	1.00	6.05	5.52	14.10	1.14	0.25	2.84	2.40
Low P	116.9	17.2	1.72	1.08	12.13	7.10	22.03	1.20	0.33	3.60	1.35
Medium P	119.0	17.3	3.94	1.63	12.77	8.18	26.52	1.10	0.45	3.25	1.50
High P	130.5	16.1	2.05	0.77	6.93	6.70	16.45	1.65	0.60	3.70	1.80

total amount of carbohydrates of the treated plants compared to their controls diminished about 1% at the high 10:1 phosphorus level and over 4.5% at the medium 20:1 phosphorus level. This would indicate either transformation to other plant constituents, translocation from the plant, or utilization as respiratory substrates.

ELEMENTAL COMPOSITION.—Without exception total phosphorus increased in the treated plants compared to the controls and the amount of increase was more than could be accounted for by the TEPP spray (table V). This increase suggests that primary absorption was influenced. Furthermore, table V showed that phosphorolysis increased in all of the sprayed plants, at all phosphorus levels, when the total phosphorus content was fractionated into the soluble and insoluble forms. Total nitrogen decreased in sprayed plants of the low and high phosphorus supply levels but total potassium increased in all TEPP-treated variables (table V). Values for total calcium increased in treated plants of the low 40:1 and medium phosphorus nutrition levels, but decreased at the high 10:1 phosphorus application. Without exception the medium 20:1 phosphorus plants, sprayed with TEPP, exhibited an increase in all fractions compared to untreated controls (table V).

RESPIRATORY MEASUREMENTS.—Young tomato plants were sprayed with TEPP at 400 and 800 p.p.m. concentrations. These concentrations were employed because growth effects and other measurements indicated that they marked the dividing line between the stimulatory and the inhibitory levels. The average value for oxygen uptake of the unsprayed controls (table VI) is slightly higher than that noted by KLINKER (**18**) for six tomato varieties, but it is believed that he used older plants in his study and hence a slower rate of respiration. Oxygen utilization expressed as percentage of the controls definitely confirmed that the 800 p.p.m. concentration inhibited respiration and that the 400 p.p.m. level greatly stimulated oxygen uptake (table VI). Although respiratory measurements were not determined for the concentrations of TEPP used below 400 p.p.m., other evidence indicates that they would have had even a greater accelerating effect upon the respiratory rate. Carbon dioxide evolution followed the same trend as for oxygen uptake at these concentrations; whereas the ratio between the two (respiratory quotient) suggests some interesting features regarding metabolism under the experimental circumstances and deserves further investigation.

Results, phase 4

ACTIVE FRACTIONS IN TEPP.—All commercial formulations of TEPP contain active and inert ingredients. These are tetraethyl pyrophosphate, which is hydrolyzed by water, the rate depending upon temperature; triethyl orthophosphate which is stable and remains in the spray unchanged; ethyl methaphosphate and the higher ethyl polyphosphates which rapidly hydrolyze to diethyl hydrogen phosphate within a few minutes (**28**). HALL and JACOBSON (**11**) present the following degradation scheme for pure TEPP:

TABLE V

RELATIONSHIP BETWEEN INORGANIC SOIL PHOSPHORUS LEVEL AND TEPP ON CARBOHYDRATES AND ELEMENTAL COMPOSITION OF TOMATO LEAVES OF PHASE 3 WHEN EXPRESSED AS PERCENTAGE OF THE DRY WEIGHT PRODUCTION. EACH TREATMENT REPRESENTS THE AVERAGE OF FOUR PLANTS SAMPLED 48 HOURS AFTER TEPP APPLICATION.

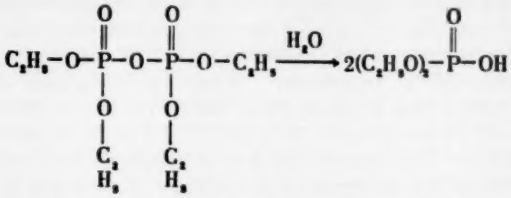
Inorganic phosphorus level	Carbohydrates					Elemental composition					
	Reducing sugars	Sucrose	Starch	Hydrolyzable residue	Total	Total N	Total K	Total Ca	Alcohol soluble P	Alcohol insoluble P	Total P
C*—Low P	1.72	1.08	12.13	7.10	22.03	1.20	3.60	1.35	0.16	0.17	0.33
T**—Low P	2.15	1.75	9.78	6.45	20.13	1.15	3.75	1.40	0.22	0.13	0.35
C—Medium P	3.94	1.63	12.77	8.18	26.52	1.10	3.25	1.50	0.15	0.30	0.45
T—Medium P	4.03	2.80	8.04	7.10	21.97	1.20	3.35	1.75	0.30	0.20	0.50
C—High P	2.05	0.77	6.93	6.70	16.45	1.65	3.70	1.80	0.30	0.30	0.60
T—High P	2.94	2.37	4.59	5.53	15.43	1.55	3.80	1.45	0.48	0.24	0.72

* Control.
** Treated.

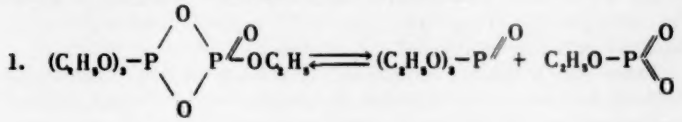
TABLE VI

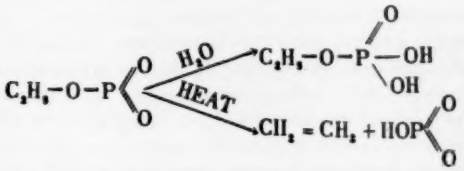
EFFECT OF TEPP ON GAS EXCHANGE IN TOMATO LEAF DISCS (O_2 UPTAKE AND CO_2 PRODUCTION). MEASUREMENTS MADE TWO HOURS AFTER SPRAY APPLICATION.

TEPP treatment in p.p.m.	Oxygen uptake—microliters/mg./hr.				Carbon dioxide output—microliters/mg./hr.				R. Q.
	First hour	Second hour	Average	Per cent. control	First hour	Second hour	Average	Per cent. control	
Control	3.13	3.00	3.06	....	2.98	3.16	3.07	....	1.00
800	2.72	2.00	2.36	77.12	2.51	2.95	2.73	88.95	1.19
400	4.78	5.15	4.97	162.41	3.49	3.06	3.28	106.84	0.66



and also show the scheme of BALAREV (3) who believed that TEPP had the structure and underwent degradation as listed below:





An experiment was set up to test some of these fractions in which young and mature tomato plants were sprayed with relatively pure TEPP at 4000 p.p.m., triethyl orthophosphate at 4000 p.p.m. and diethyl hydrogen phosphate. The diethyl hydrogen phosphate was obtained by hydrolyzing pure TEPP with 20 times its volume of water for one week at 30° C.

Within two hours or less the TEPP-sprayed plants were manifesting a number of effects: severe epinasty was apparent, and leaves had become chlorotic, and the growing points were twisted and dried. In four hours' time the young plants appeared to be dead while the old plants were exhibiting severe symptoms. At the end of 24 hours, all TEPP-treated plants were dead. The plants treated with the diethyl-hydrogen-phosphate fraction were not immediately affected except to show slight epinasty and the usual growing point symptoms. These effects, at the time, were thought to be due to incomplete hydrolysis of the original TEPP. Observations of the young DEHP-sprayed plants to maturity (fig. 4 F), however, showed that this was not the entire explanation, for these plants displayed the severe formative symptoms, typical of 2,4-D injury, over a longer period of time. The triethyl phosphate-treated series exhibited little effect except burning and chlorosis of the foliage. The responses of the mature plants of this experiment are illustrated in figure 4 E.

COMBINED EFFECTS OF TEPP AND 2,4-D.—To determine if the effects of

TEPP and 2,4-D were additive, four lots of young vegetative tomato plants consisting of four plants to each lot were sprayed with 800 p.p.m. TEPP, 10 p.p.m. 2,4-D, 10 p.p.m. 2,4-D plus 800 p.p.m. TEPP, and 100 p.p.m. 2,4-D plus 800 p.p.m. TEPP, respectively. Within two hours after application, the plants treated with 10 p.p.m. 2,4-D alone were only slightly epinastic, whereas in the plants receiving 800 p.p.m. TEPP alone as well as in both combined levels of 2,4-D plus TEPP treated plants, the usual epinastic and mottled chloroses were apparent. Five days later the 10 p.p.m. 2,4-D plants had mostly recovered from the earlier symptoms but in the 800 p.p.m. TEPP plants death of the growing points was noted. The combined 10 p.p.m. 2,4-D and 800 p.p.m. TEPP-treated plants externally appeared to be dead. The 100 p.p.m. 2,4-D plus 800 p.p.m. TEPP-treated plants were definitely dead and extremely malformed and twisted.

Tests for ethylene evolution.—Enclosure of the TEPP-sprayed plants in desiccators with etiolated pea seedlings up to 72 hours resulted in some indication that ethylene was produced. Although not conclusive, it is possible that some of the ethylene inhibition symptoms exhibited by the seedlings when enclosed with the sprayed plants were due to normal metabolism of plants and not to TEPP degradation. However, inhibition of the seedlings enclosed with the unsprayed tomato plants was much less severe than it was when the seedlings were enclosed with TEPP-sprayed plants. The treated tomato plants themselves, however, were severely epinastic, chlorotic, and apparently dead at the higher concentration of TEPP. The pea seedlings confined with a hydrolyzing sample of TEPP were comparable to the controls and confirms that ethylene is not a direct hydrolytic product to TEPP.

Results with ATP.—Thirty days after the young tomato plants had been sprayed with adenosine triphosphate only slight indications of formative effects were apparent. These symptoms when present largely resembled those elicited by 200 p.p.m. TEPP (fig. 3 C). Some burning and chlorosis of foliage also followed spraying. Compared to the controls the ATP-sprayed plants were slightly stimulated in growth as shown by somewhat heavier foliage and greater shoot length. The plants receiving the soil application of ATP however were essentially the same as the controls.

Discussion

The fact that all degrees of formative response, ranging from stimulation to complete inhibition and death was obtained, depending upon the concentration of TEPP employed, indicated that TEPP or its degradation products possessed definite growth-regulating properties. The data for stem elongation and flowering (table II) showed that approximately the same concentrations of TEPP stimulated or inhibited these divergent processes. This is not surprising as Leopold and Thimann (**21**) have shown that concentrations of indoleacetic acid that favored stem growth also favored flowering. Smith *et al.* (**26, 27**) have reported that in roses increased vigor, as

shown by a deeper green color, larger leaves, longer and heavier stems, more and larger flowers, followed the use of HETP. Their data showed that the average stem length of cut roses increased three to six inches and flower production by 10 to 30% compared to untreated plants and led these workers to suggest that stimulatory action, beyond that explained by the elimination of spider mites, resulted from the use of HETP.

The lowered dry weight and carbohydrate content of the TEPP-treated plants, in addition to accelerated hydrolysis (or phosphorolysis) revealed that the primary effects of TEPP on plant metabolism was the promotion of enzymatic and respiratory action at the stimulatory concentrations but inhibition of these processes at the retarding levels. The stimulation and inhibition effects of TEPP on respiration were further confirmed by the Warburg determinations (table VI). Therefore, it would seem that the most logical explanation of the mode of action of TEPP would be found in its effects on the enzymatic system of the plant. HALL (**10**) implied that the toxicity of these compounds on animals and insects was manifested through the inhibition of the acetyl choline esterase system. ADLER *et al.* (**1**) believed that in animals the addition of pyrophosphates inhibited isocitric dehydrogenase by the removal of Mg^{++} or Mn^{++} which are required for the activity of coenzyme II. This interpretation, however, is questioned by KREBS (**19**). It has been observed that the energy liberated by the action of succinic dehydrogenase can be utilized for the synthesis of energy-rich phosphate bonds (**5**). The inhibition of succinic dehydrogenase, known to function both in the respiration of plant and animal cells, was accomplished in two steps (**20**). HCN inhibited only the ability to utilize oxygen and not the ability to decolorize methylene blue, but the last step was inhibited by selenite, arsenite, malonic acid, and pyrophosphate (**20**). The inhibition of yeast aldolase but not muscle aldolase by pyrophosphate has also been reported (**31**).

It is also known that ethylene, both at the concentration of 1 p.p.m. and 1000 p.p.m. causes the simple soluble substances of plants to increase at the expense of the higher soluble and insoluble forms (**13, 14, 15, 16**). Ethylene, 1 p.p.m. in air, increased the respiration of potatoes in storage (**16**) and HANSEN (**12**) has confirmed this effect of ethylene on fleshy fruits. However, the pea test, to date, has not demonstrated conclusively that ethylene originated from degradation of the organic phosphorus compounds, even though it is possible for it to arise from thermal breakdown of ethyl metaphosphate at temperatures above 140° C (**11**). A personal communication from Dr. S. A. Hall, of the Bureau of Entomology and Quarantine, Beltsville, Maryland, also stated that they have never been able to demonstrate ethylene evolution from HETP or TEPP, even when sprayed on hot steam pipes in the greenhouse. The fact that the TEPP (or degradation products) stimulus persists in the soil, and that plants show formative effects, at least two months after its addition to the soil, still remains to be satisfactorily explained. Perhaps, it is possible by some unknown mechanism, for ethyl-

ene to be evolved from TEPP-degraded products as diethyl hydrogen phosphate or diethyl metaphosphate. ZIMMERMAN and HARTZELL (**34**) arrived at approximately this conclusion in their study.

The formative responses and metabolic effects of TEPP on plants closely coincide with those induced by 2,4-D. KELLEY and AVERY (**17**) have shown that 2,4-D has both stimulatory and inhibitory effects on the respiration of pea and oat tissues. A concentration of 1 gram per liter (1000 p.p.m.) was inhibitory; concentrations of 100 to 200 mg. per liter (100 and 200 p.p.m.) and lower, stimulated oxygen uptake in oat tissue, and 10 mg. per liter and lower were stimulatory to pea tissue. These workers reported that the maximum stimulation of respiration approximated 50%, whereas the maximum stimulation in the present study for the concentrations of TEPP employed was 62%. MCILRATH (**24**) reported that in cotton, using formative effects as the index, low carbohydrate plants are most susceptible to TEPP and HETP. His report was in accord with KELLEY and AVERY's work (**17**) showing that 2,4-D brought about greater stimulation of respiration in starved than unstarved oat tissue.

The elemental composition of plants, both in the first experimental phase conducted for 30 days and the 48-hour experiments showed increased tissue phosphorus in the TEPP-sprayed plants. In some cases the amount was more than could be accounted for by the phosphorus from the TEPP application. The general increase of the elements potassium and calcium in the treated plants, even during the relatively short 48-hour experiment (table V), and the correlation with the respiratory stimulation obtained at 400 p.p.m. level (table VI) suggested increased root absorption and additional support for the hypothesis that respiratory energy is utilized in primary absorption.

The similar, if not identical, formative effects produced by TEPP and 2,4-D and their mutual effect on respiration suggests a common mode of action of the two compounds on plant metabolism, possibly through the effect on aerobic respiration. LIPMANN (**22**) reported that the one known mechanism for the utilization of energy liberated by the metabolism of foodstuff is through the formation of labile high-energy phosphate bonds. LIPMANN (**22**) has estimated that the free-energy decrease of these compounds upon hydrolysis is of the order of magnitude of 10,000 to 12,000 calories per mole. One of the high-energy phosphate linkages known in biological systems is the bond between phosphate and other phosphate groups as in the general type —P—O~P—. This type of linkage exists in tetraethyl pyrophosphate (see structure on page 517). However, little work apparently has been done on the nature of the energy relations of the phosphorus-oxygen bonds of TEPP. HALL and JACOBSON (**11**) did calculate the Arrhenius heat of activation, which should be a measure of the energy to break the P—O—P linkage by hydrolysis, to be 10,300 calories per mole. This value agrees with the energy necessary for the high-energy phosphorus-bond type of compound given by LIPMANN (**22**). The rapidity of hydrolysis also indicates that TEPP possesses properties of this type of compound. In his explana-

tion of the mode of action of the 2,4-D hormone-type phytocide, VAN OVERBEEK (**30**) postulated that, after combining with suitable proteins, 2,4-D stimulated the liberation of organic phosphate from phosphorylated compounds. The lethal effect of 2,4-D, according to MCELROY (**23**) possibly might be due to greatly stimulated glycolysis and that the oxidative processes concerned in synthesis may not be able to compete with the available hydrogen acceptors and are consequently inhibited.

The data of this study suggest that TEPP functions in reverse to the above hypothesis for 2,4-D, serving either directly as a high-energy phosphorus substrate for transphosphorylation or indirectly in stimulating the natural synthesis of high-energy phosphorus bond compounds by the plant with subsequent liberation of energy for growth. The latter perhaps appears more logical in light of the results obtained with ATP spray. Thus both 2,4-D and TEPP may have the same net result on plant metabolism although effecting this response by different biological pathways.

The increased effect of TEPP plus 2,4-D, contrasted to either compound alone, lends support to this hypothesis. In addition, phosphorolysis was accelerated in the TEPP-treated plants as evidenced by the accumulation of inorganic phosphorus in these plants. If further research substantiates this explanation of hormone action and respiration, some of our present theories must be re-evaluated accordingly.

Summary

1. Morphological, physiological, and metabolic effects produced by the organic phosphorus insecticides, hexaethyl tetraphosphate and tetraethyl pyrophosphate were studied singly and in combination with inorganic soil P in carnation and tomato.

2. The abnormal flowering symptoms first observed in carnation were reproduced in this species by aqueous spray applications of hexaethyl tetraphosphate containing the active constituent tetraethyl pyrophosphate and the symptoms are described and illustrated.

3. Stimulatory, inhibitive, and lethal responses of tomato to TEPP were noted and the concentration ranges of TEPP necessary to produce these were reported. Formative effects, even at extremely high soil levels of superphosphate, were not produced by inorganic phosphorus applications.

4. Of the concentrations tested 400 p.p.m. of TEPP marked the upper limits of stimulation for stem elongation and time of flowering in tomato. The difference in the number of flowers produced was not considered to be significant, however, in this study.

5. Weight production and carbohydrate content decreased in treated plants. Hydrolysis of reserve carbohydrates and the accumulation of soluble sugars were accelerated by TEPP.

6. Respiratory measurements showed that 400 p.p.m. of TEPP greatly accelerated oxygen uptake (62% above the controls), but 800 p.p.m. inhibited oxygen uptake (27% of controls).

7. Phosphorus fractionation disclosed that TEPP increased soluble phos-

phorus at the expense of the insoluble fraction, and indicated accelerated phosphorolysis in the treated plants.

8. The elements K, P, and Ca increased in plants treated with 400 p.p.m. TEPP. These changes are interpreted to mean a stimulation of primary absorption occurred due to the increased respiratory energy resulting from treatment.

9. The similarity in morphological and metabolic responses of plants to 2,4-D and TEPP has been noted and a common mode of action of the two compounds discussed.

The writer is gratefully indebted to Mr. Harry C. Lane for his technical assistance in helping conduct the Warburg measurements, to Dr. Frank M. Eaton, BPISAE, USDA and Texas Agricultural Experiment Station, and Dr. H. E. Joham and Dr. G. M. Watkins of the Department of Plant Physiology and Pathology for their helpful comments during the preparation of the manuscript. Acknowledgment is also due Monsanto Chemical Company of St. Louis, Missouri, and California Spray Corporation of Richmond, California, for kindly supplying the TEPP used in this work.

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TRANSLOCATION OF FOOD RESERVES FROM SOYBEAN COTYLEDONS AND THEIR INFLUENCE ON THE DEVELOPMENT OF THE PLANT[1]

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(WITH SEVEN FIGURES)

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Introduction

This study was initiated to determine the quantitative relationships between the various reserve constituents in soybean seeds as they are used by the developing seedling, and the effect of removal of the food supply contained in the cotyledons on subsequent growth of the plants. Many investigations have dealt with the transformations which occur in the reserve food materials in seeds of crop plants as germination proceeds. Few studies, however, have been concerned with the rate of transfer of these foods to the seedling. No reports have been found in the literature on how long the seedling is dependent on the reserves in the cotyledons for successful establishment and later development. This information was desired for use in mineral nutrition studies on soybeans particularly where the effects of limiting levels of some of the major nutrients are being investigated. At starvation levels of nutrition, it was felt that the amount of a particular element supplied by the cotyledons may be sufficient to influence experimental procedure.

Using semiquantitative, microchemical methods on freezing microtome sections of Manchu soybean seeds, VON OHLEN (**12**) followed the translocation of organic and inorganic substances from the cotyledons to the seedling during germination in the dark. His results show that oil moved out of the cotyledons more rapidly than the other main organic reserve, protein, and that among the mineral elements, potassium was translocated at a greater rate than phosphorus or magnesium. HOLMAN (**7**) found that the fat content of Agat(e) soybeans had decreased 78%, 17 days after planting. The work of MILLER (**10**) on sunflower probably gives the most complete data on the transformation and movement of foods in oilseed plants. The seedling material analyzed was grown under greenhouse conditions with natural light but at emergence the seedlings were transferred to a carbon dioxide-free atmosphere so that the seedlings were dependent upon the reserves of the cotyledons throughout the growing period. Miller's data show that hydration of the cotyledons was complete in 10 days, dry weight decreased gradu-

[1] Contribution No. 210 from the U. S. Regional Soybean Laboratory, Division of Forage Crops and Diseases, Bureau of Plant Industry, Soils and Agricultural Engineering, Agricultural Research Administration, U. S. Department of Agriculture, Urbana, Illinois.

ally throughout the 13-day germination period, ash content decreased during the first week and then increased, and the ether extract decreased more rapidly and to a lower relative level than protein.

The translocation of organic and mineral reserves from wheat seed to the seedling was investigated by YOCUM (**14**). He found that nearly all of the total nitrogen was removed from the seed within 12 days after planting, total sugars increased rapidly for six days and then decreased to nearly zero by the 12th day. Ether extract of the seed decreased slowly throughout the 25 days of the experiment, starch decreased to nearly zero at 12 days and about two thirds of the ash constituents were removed in nine days with little change during the remaining 16 days.

Materials and methods

Two yellow-seeded, commercial-type soybean varieties, Lincoln and Earlyana, were used in this study. Lincoln is the most widely grown of the soybean varieties in the Midwest and produces a seedling of average vigor. The variety Earlyana normally produces a more vigorous seedling than Lincoln. The difference in seedling vigor between these two varieties is usually observable only during the early stages of development.

Two plantings were made, one under field conditions and the other in sand in the greenhouse. The field planting consisted of four replications of paired, 10-row plots of each variety. The rows were 19 feet long and spaced 24 inches apart. At the time of cotyledon removal, the plants were thinned to a spacing of approximately two inches. In the greenhouse planting, alternate rows were planted of the two varieties at a depth of one inch.

Cotyledons were removed from seedlings in the greenhouse planting at 24-hour intervals until emergence, 96 hours after planting. The seedlings from the greenhouse planting were discarded after removal of the cotyledons. In the field planting, rows were selected at random in each of the 10-row plots for each of the cotyledon removal treatments. Cotyledons were removed from the field planting at emergence and at 2, 4, 6, 9, 13, 17, and 28 days after emergence. Some of the cotyledons were turning yellow 17 days after emergence. At the time the last collection was made, in order to obtain enough cotyledons for a sample, it was necessary to remove all attached cotyledons from two rows in each block. Table I shows the time intervals between cotyledon removals and the stage of development of the plants at the time the cotyledons were removed.

Upon removal from the seedlings, the cotyledons were placed in airtight containers, taken to the laboratory, weighed and dried in a forced draft oven at 90° C. In addition to moisture content of each collection, dry weight determinations of 200 cotyledons were made prior to grinding the samples for chemical analysis. All of the chemical analyses are reported on the basis of composition of 200 cotyledons or 100 seeds rather than on a percentage basis. Since it was considered unlikely that all food materials in

TABLE I

COTYLEDON REMOVAL INTERVALS AND SIZES OF SEEDLINGS WHEN COTYLEDONS WERE REMOVED.

Collection no.	Days after planting or emergence	Degree of plant development at cotyledon removal
1	0	Dry seeds
		Greenhouse Planting
2	1	Radicles* ¼ inch long
3	2	Radicles 1¾ inches long
4	3	Radicles 4 inches long
5	4	Radicles 6 inches long, cotyledons emerging
		Field Planting
6	0	Emergence (10 days after planting), cotyledons turning green
7	2	Seedlings 1 to 1½ inches tall, unifoliate leaves unfolding
8	4	Seedlings 2 inches tall
9	6	Seedlings 2 to 3 inches tall
10	9	Seedlings 3 to 4 inches tall, 1st trifoliate leaves unfolding, cotyledons fully expanded
11	13	Seedlings 4 to 5 inches tall
12	17	Seedlings 5½ to 7 inches tall, 2nd trifoliate leaves unfolding
13	28	Lincoln seedlings 12 to 13 inches tall, Earlyana 14 to 15 inches, 4 trifoliate leaves, cotyledons turning yellow and dropping

* Hypocotyl and root.

the cotyledons would be used up at the same rate, the former method of reporting chemical composition was preferred in this instance.

Unless otherwise indicated, the approved methods of the ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS (**2**) were used in the chemical analyses. Macro-methods were followed wherever possible. It was necessary to use micro-methods on some of the samples collected after most of a particular substance had been removed from the cotyledons. Protein was determined by the Kjeldahl-Gunning-Arnold method using 6.25 as the nitrogen-to-protein factor (**2**, p. 26). Sugars were extracted (**2**, p. 409) and inverted (**2**, p. 566) and analyzed by the Scales (**2**, p. 571) or the Somogyi (**2**, p. 578) method. Ether extract was determined using Butt extraction apparatus with petroleum ether as a solvent (**1**) and the iodine number of the extract by the Wijs method (**1**). Potassium was determined gravimetrically (**2**, p. 121) or colorimetrically by WANDER'S (**13**) method. Phosphorus determinations were made volumetrically (**2**, pp. 22, 23). Magnesium was separated (**6**) and determined gravimetrically (**2**, p. 120). Calcium was determined volumetrically (**2**, pp. 119, 120).

The effects of cotyledon removal on the growth and development of the plants were observed in the field planting. Comparisons were made during

the growing season between the controls and treated plants as to their general appearance, bloom dates, height, date of maturity, and seed yield. Seed yields were based on the amount of seed produced by the plants in a 1-rod section of the 19-foot rows.

Results

Changes in composition in the cotyledons

The data from both greenhouse and field plantings are presented in the same graph for each of the factors considered. The time from planting to emergence was four days for the greenhouse planting and 10 days for the field planting. Thus, the rate of development of the seedling until emergence was two and one-half times more rapid in the greenhouse than in the field. The sand temperature was 22 to 24° C compared with an average temperature of 17° C for the field soil one inch below the surface.

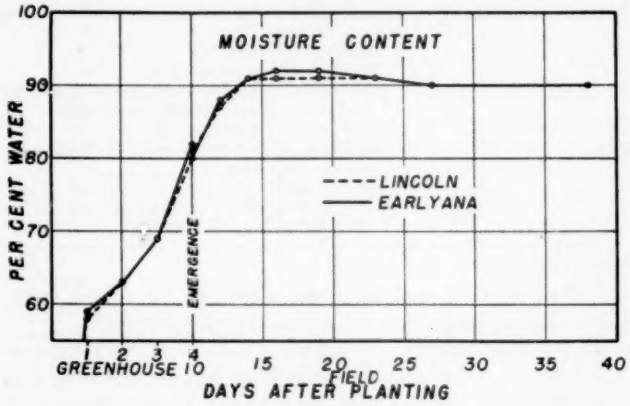

Fig. 1. Moisture content of samples of cotyledons of Lincoln and Earlyana soybeans from planting until senescence (28 days after emergence in the field planting).

Moisture content.—The changes in moisture content of the cotyledons of the two soybean varieties during the period of sampling are shown in figure 1. The curves indicate little difference between varieties. Within 24 hours after planting, the moisture content of the cotyledons had risen from the 13% moisture content of the dry seeds to 58 and 59% respectively, for Lincoln and Earlyana. The increase in hydration was less rapid from that point but continued at a fairly even rate, until a moisture content of 91% had been reached four days after emergence. At emergence, in both greenhouse and field plantings, the moisture content was 80% or slightly above. No significant changes in hydration of the cotyledons were found in samples collected between four and 28 days after emergence.

DRY WEIGHT.—The curves in figure 2 show the rates of decrease in dry weight in the cotyledons. The original weight of 200 oven-dry cotyledons was 14.32 gm. for Earlyana and 12.42 gm. for Lincoln. There was a decline in dry weight until nine days after emergence in both varieties, after which time dry weight remained relatively constant. Approximately half of the decrease in dry weight occurred during the interval from planting to emergence. The larger seed of Earlyana was reflected in a slightly greater weight in the cotyledons at all sampling intervals. The weight of 200 cotyledons was 4.32 gm. (Earlyana) and 3.75 gm. (Lincoln) 28 days after emergence, a total decrease of 70% for each variety.

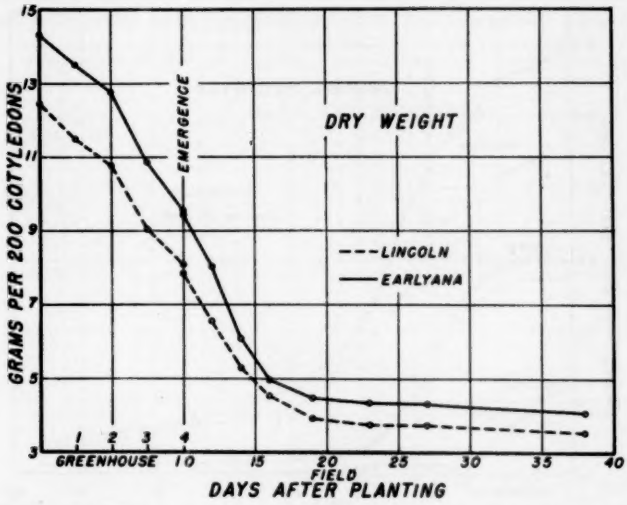

FIG. 2. Changes in dry weight of cotyledons of Lincoln and Earlyana soybeans from planting to senescence.

ORGANIC FOOD RESERVES.—The curves in figure 3 present the results of the analyses for protein, ether extract and sugars in the cotyledons of Earlyana and Lincoln at the various intervals of collection. These three classes of food reserves represented 69.6% of the total dry weight of the Lincoln seeds, 69.1% of the Earlyana seeds, and are also the most readily available sources of energy and other nonmineral supplies for the seedling.

The results of the protein analyses (fig. 3) showed that there was a rapid decrease in protein content in both varieties from planting time until nine days after emergence followed by a steady but much less rapid loss until the end of the collection period. Of the 4.67 gm. per 200 cotyledons total protein in Lincoln, 69% remained at emergence, 20% nine days later, and 7% at 28 days. The same number of cotyledons of Earlyana contained

6.00 gm. of protein before planting of which 71% remained at emergence, 30% nine days later and 7% 28 days after emergence.

Somewhat in contrast to the behavior of the proteins, the ether-extractable substances decreased very little during the first two days in the cotyledons of the greenhouse planting. The period of rapid loss of the oil-like fraction extended from the second day after planting until six days after emergence. After this time there was little change in the ether extract content of the cotyledons. The original ether extract of 200 cotyledons was 2.73 gm. and 2.95 gm. for Lincoln and Earlyana, respectively. At emergence approximately half of these substances remained in both varieties.

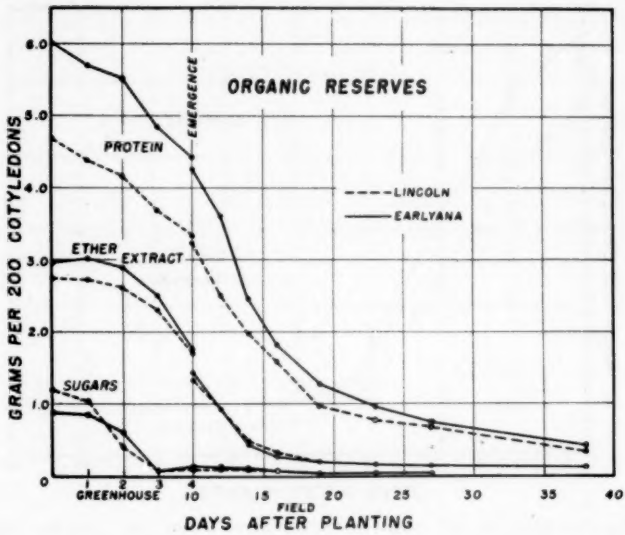

FIG. 3. Protein, ether extract and sugar content of cotyledons of Lincoln and Earlyana soybeans from planting to senescence.

Only one tenth of the ether extractables were contained in the cotyledons six days after emergence and only 4% 22 days later.

The iodine number of the ether extract of the cotyledons from ungerminated seeds was 134 for Lincoln and 132 for Earlyana. There was no consistent change in these values in subsequent samples of either variety.

Compared with the other organic constituents, the total sugars present in the cotyledons made up a much smaller fraction. Before germination, 200 cotyledons of Lincoln contained 1.19 gm. of sugar compared with 0.94 gm. in Earlyana cotyledons. The sugars decreased rapidly, until only a trace remained one day before emergence. Less than 10% of the total sugars were reducing sugars in the ungerminated seeds. The amount of

reducing sugars varied between 0.11 and 0.03 gm./200 cotyledons in Lincoln and 0.10 and 0.03 gm./200 cotyledons in Earlyana, with the smaller amounts in each instance occurring in the field samples collected 17 days after emergence. The behavior of the sugar components was much the same as has been noted elsewhere for this (**7, 11**) and other oilseed plants (**8, 10**).

MINERAL RESERVES.—The changes in potassium content in the cotyledons are shown in figure 4. The curve for the cotyledons of Lincoln shows a rapid loss of K from planting until emergence and then a gradually decreasing rate to the final collection. Earlyana cotyledons lost only a very small amount of K during the first day after planting but after two days the rate of decrease was similar to that shown in the first day for Lincoln and continued at this rate until the sixth day after field emergence. The slight

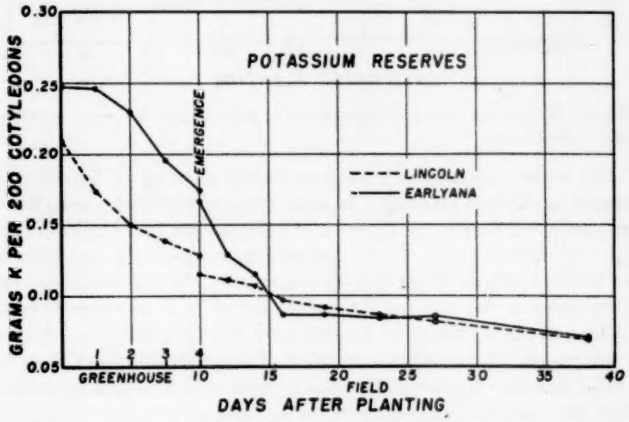

FIG. 4. Potassium content of cotyledons of Lincoln and Earlyana soybeans from planting to senescence.

decrease in K noted in later samples was also similar to that observed in Lincoln cotyledons. The K contained in 200 cotyledons of Earlyana before planting was 0.247 gm. and 0.070 gm. (28%) remained 28 days after emergence. Of the 0.177 gm. translocated to the seedling, 44% had been translocated by the time of emergence and 90% within six days after emergence. Two hundred cotyledons from ungerminated Lincoln seed contained 0.210 gm. of K and of this amount 0.069 gm. (33%) remained in the cotyledons 28 days after emergence. Sixty-two per cent. of the mobile 0.141 gm. had been translocated to the seedling by the time of emergence and 80% six days later.

The phosphorus content in milligrams per 200 cotyledons at the 12 sampling intervals is presented in figure 5. Both varieties show a maximum rate of transfer of this element from the cotyledons to the seedlings from

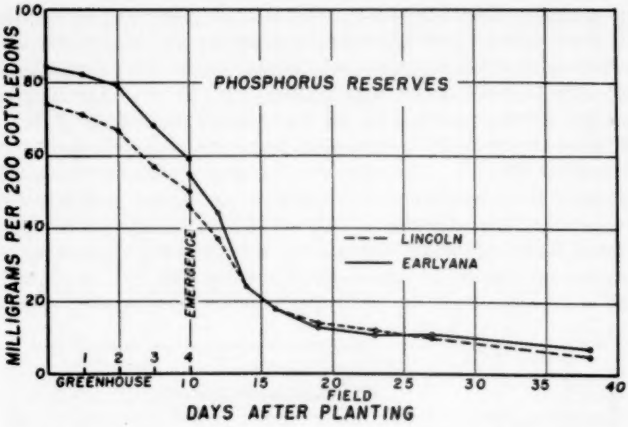

FIG. 5. Phosphorus content of cotyledons of Lincoln and Earlyana soybeans from planting to senescence.

two days before emergence in the greenhouse planting to four days after emergence in the field planting. In ungerminated Earlyana seeds, 200 cotyledons contained 84 mg. of P and a similar sample of Lincoln cotyledons 74 mg. Of these amounts, 77 mg. and 69 mg., respectively, had been transferred to the seedlings 28 days after emergence. As the seedlings emerged from the sand or soil 40% of the mobile P had been transferred from the cotyledons to the seedlings in Lincoln and 37% in Earlyana. Four days after emergence the rate of translocation of this element began to diminish. The total amount was about the same in both varieties, Lincoln cotyledons had lost 78% and Earlyana 81% of the mobile P.

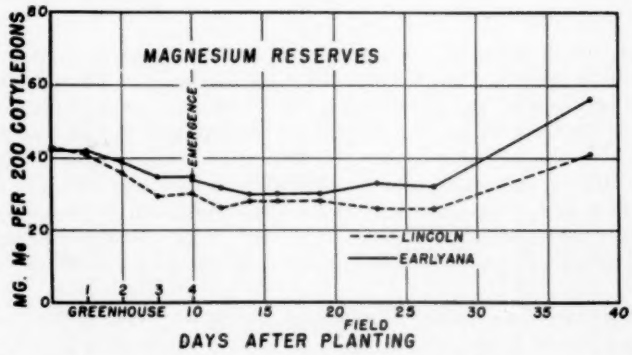

FIG. 6. Magnesium content of cotyledons of Lincoln and Earlyana soybeans from planting to senescence.

The curves in figure 6 show that only a very small quantity of magnesium was moved from the cotyledons to the seedlings in either variety. There was a gradual loss of Mg during the first three days of the greenhouse germination period, 42 to 29 mg. for Lincoln, 43 to 35 mg. for Earlyana, and from that time on the only change of any magnitude recorded was in the last sample collected. In the final samples, the Mg content in the cotyledons was nearly as high or higher than in the original samples from ungerminated seeds, 41 mg. for Lincoln and 56 mg. for Earlyana.

The behavior of calcium in the cotyledons is shown in figure 7. The curves for both varieties indicate little if any transfer of calcium from the cotyledons to the seedlings. As soon as emergence had occurred, the cotyledons apparently began absorbing calcium from the seedling. The calcium

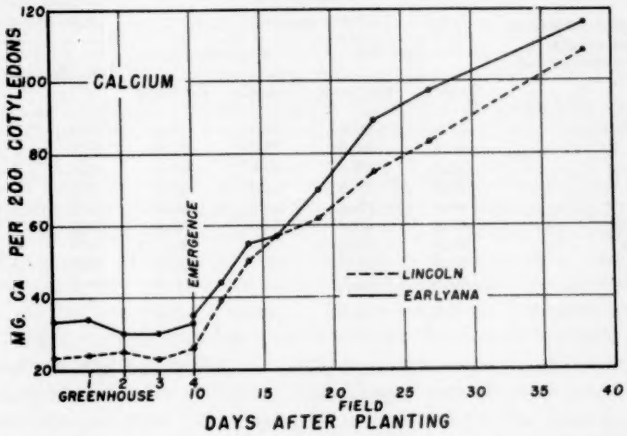

FIG. 7. Calcium content of cotyledons of Lincoln and Earlyana soybeans from planting to senescence.

content of 200 cotyledons of Earlyana was 33 mg. in ungerminated seeds, 35 mg. at emergence, and 116 mg. 28 days later. In the Lincoln cotyledons there were 23 mg. of calcium in dry seeds, 30 mg. at emergence and 108 mg. 28 days later.

RESPONSES OF THE PLANTS TO COTYLEDON REMOVAL

One of the effects of cotyledon removal on the plants in the field was a temporary yellowing of the seedlings of both varieties in the rows where the cotyledons had been removed at emergence, and at two and four days later. The younger the seedlings at the time of cotyledon removal, the more noticeable the chlorosis. Since this response was not observed in seedlings whose cotyledons were removed six days or more after emergence, or in the control plants, it could hardly be ascribed to environmental effects. Where the

yellowing occurred, all leaves were involved. The chlorotic appearance persisted only during a period of 10 to 15 days.

Time of cotyledon removal had no effect on the date of blossoming in either of the varieties. The only lasting effect of cotyledon removal on the development of the plants was a reduced plant height from certain treatments. Height measurements were made on the field planting on July 7, when the plants of both varieties were in a vegetative condition, and on September 23, at maturity. These data are summarized in table II.

TABLE II

EFFECT OF COTYLEDON REMOVAL ON PLANT HEIGHT AND SEED YIELD IN LINCOLN AND EARLYANA SOYBEAN VARIETIES.

Time of cotyledon removal (Days after emergence)	Plant height (centimeters)				Seed yield (gm./rod-row)	
	July 7		September 23			
	Lincoln	Earlyana	Lincoln	Earlyana	Lincoln	Earlyana
Emergence	27.5	26.6	74.3	70.5	552	433
2	30.5	34.0	78.8	77.5	597	474
4	36.9	39.9	80.8	80.5	655	530
6	40.0	42.4	81.5	80.0	712	485
9	40.4	45.4	81.5	80.8	672	472
13	40.4	45.4	83.3	81.8	662	492
17	42.0	44.6	82.8	82.0	609	565
28	43.0	44.8	83.0	81.0	653	539
Control	40.0	46.1	82.0	80.8	645	481
L.S.D. 5% level	3.3	4.0	3.4	2.4		

The average height of Lincoln plants on July 7 was 12.5, 9.5, and 3.1 cm. less in the first three treatments, respectively, than the plants in the control rows which were 40.0 cm. in height. For Earlyana, the average plant height at this date for the first four intervals of cotyledon removal was 19.5, 12.1, 6.2, and 3.7 cm. less, respectively, than the 46.1 cm. height for the control rows. Measurements made at maturity showed shorter plants only for the rows of both varieties where the cotyledons had been removed from the seedlings at emergence or two days later. The average heights of the mature plants of Lincoln in the first two treatments were 7.7 and 3.2 cm. less than untreated plants (82.0 cm.). The plants in the first two treatments for Earlyana were 10.3 and 3.3 cm. shorter than the control plants (80.8 cm.). The differences in height referred to above met the requirements for significance (analysis of variance) at the 5% level except the differences in height between the control rows of Lincoln and the third treatment (four days after emergence) in the July 7 measurements and the second treatment at maturity where the values missed being statistically significant at this level by a very small margin.

The average seed yield for rod rows of each treatment of the two varieties is presented in table II. Analysis of variance of the yield data indicated

that the variability was too great to meet the requirements for significance at the 5% level. Since in each variety, however, the average yield for the rows having the cotyledons removed at emergence was lower than any of the other treatments or control, it is possible that the decrease was a result of the treatment. The mean yield for Lincoln where the cotyledons were removed at emergence was 552 gm. compared with 645 gm. for the untreated rows, and the same comparison in Earlyana showed yield values of 433 gm. and 481 gm., respectively. With the possible exception of the 2-days-after-emergence treatment in Lincoln, where the decrease in yield may have been a treatment effect, none of the other yield means suggest that cotyledon removal had any effect on seed production.

Discussion

Both dry weight and moisture content gave an index of the rate of movement of food reserves from the cotyledons to the seedling. With the exception of the rapid initial water uptake the first day, the change in moisture content was inversely proportional to the dry weight. A similar relationship between these two factors was found in sunflower (**10**) and wheat (**14**) cotyledons.

Total loss of dry weight of the cotyledons in this study was 70% for both varieties 38 days after planting. Sunflower cotyledons (**10**) on plants which were grown in the light but not allowed to carry on photosynthesis, lost 65% of their original weight in 13 days. Cotyledons of garden beans lost 58% and corn only 26% of their original dry weight when seedlings were grown to exhaustion in distilled water (**3**). Wheat cotyledons (**14**) lost 91% of their original weight 25 days after planting. The wheat seedlings were allowed to develop normally and so these data are more comparable with those reported here for soybeans. The difference between 91% loss in dry weight for wheat cotyledons compared with 70% for soybean cotyledons, is probably due to the fact that the cotyledon of wheat seeds serves only as a source of reserve food for the seedling while soybean cotyledons carry on photosynthesis soon after emergence. It is also probable that micro-biological activity played a part in removing the stored food in the wheat cotyledons in the later stages of germination since Yocum apparently did not sterilize either seeds or soil.

The rapid decrease observed in the nitrogenous material in the cotyledons during the early stages of germination is at variance with what has been observed in other plants, where the changes were gradual (**10**), relatively small (**14**), or practically none (**8**). Von Ohlen (**12**) observed a decrease in protein concentration in soybean cotyledons beginning with the fifth day after planting. Since the protein concentration in soybean cotyledons is very high, it is doubtful that Von Ohlen could detect the initial changes in protein concentration with the technique he used.

The lag observed in the disappearance of the ether-extractable substances from the cotyledons during the first stage of germination followed by a rapid

loss during the emergence period agrees with the work of other investigators (**5, 7, 11, 12**) on this phase of soybean development. There is little agreement, however, on what has been found concerning changes in the iodine number of the ether extract of soybean cotyledons during germination. EYSTER (**5**) and MACLACHLAN (**9**) observed no change in the iodine number as is reported here, while others have found either a decrease (**7**) or an increase (**11**). There was no change in the iodine number of the oil in germinating Tung kernels (**8**) while a decrease of 69 units was reported in the oil of sunflower cotyledons (**10**). The soybean seedlings depended to different degrees on the supplies of K, P, Mg, and Ca available in the cotyledons. Averages of the data for the two varieties show that 69% of the total K and 92% of the total P in 200 cotyledons was transferred to the seedlings. There was, however, a net gain of 4% in Mg and 300% in Ca at senescence compared with the original amounts present. Calcium possibly failed to move readily from the cotyledons as a result of being present in a relatively insoluble form in the cells and/or because it could be easily absorbed from the soil. No reasons for the differential movement of the mobile elements K, P, and Mg were apparent unless the seedlings were able to absorb Mg from the soil more readily than from the cotyledons while the reverse was true for K and P. The ease with which K may be translocated from soybean cotyledons was also shown in VON OHLEN'S (**12**) experiments. Potassium was observed to decrease to zero 25 days after planting, while P and Mg decreased to about one half of their concentration in ungerminated seed. With mineral-starved seedlings, translocation of one half or more of the original Ca from the cotyledons to the seedlings has been observed for garden beans, corn (**3**) and jack beans (**4**).

The translocation of both organic and inorganic substances from the cotyledons to the seedlings was accomplished in both varieties used in this study in about the same time interval. At the later dates of collection of samples, 200 cotyledons of each variety contained about the same total quantities of the substances studied. Since Earlyana cotyledons contained larger quantities of the substances to be transferred than those of Lincoln, because of the larger seed size of the former variety, the rates of transfer of food reserves were slightly higher for Earlyana than for Lincoln. An exception to this behavior occurred in the movement of K from the cotyledons. This element moved rapidly out of Lincoln cotyledons with no observable lag phase while in Earlyana cotyledons, K followed the more common pattern of a gradually accelerating rate of transfer with a maximum near emergence.

The transition in function from that of storage organs to leaves carrying on the normal processes of nonspecialized leaves was accomplished in a relatively short time by the cotyledons of soybeans. The data presented explain why there was little effect on subsequent development of the plants when cotyledons were removed later than four days after emergence in the field planting. Removal of cotyledons at emergence resulted in a stunting effect

from which the plants never fully recovered (table II). The stunting of the plants was probably associated with the same temporary deficiency which caused chlorosis in the seedlings whose cotyledons were removed at emergence or soon after. Although many factors could have been involved, the general nature of the chlorosis suggested the effects of lack of nitrogen. A similar yellowing is noted occasionally in normal seedlings in the field when weather conditions result in lack of nitrogen in the soil before nodules are fully developed on the roots. It is doubtful, then, that early removal of cotyledons from soybean seedlings could serve a useful purpose in nutrition studies since abnormal plants would be produced. If cotyledon removal was delayed until normal plants could be produced, most of the reserves would have been translocated to the seedling and there would be little gained by the effort.

Summary

The following results were obtained in a study of (a) the changes which occurred in the cotyledons of Lincoln and Earlyana soybeans from planting to cotyledon senescence and (b) the effects of cotyledon removal on development of the plant:

1. Hydration of the cotyledons was complete four days after emergence.

2. Total decrease in dry weight of the cotyledons was 70%. One half of this decrease had occurred by emergence.

3. Of the organic reserves, the sugars disappeared most rapidly, only a trace remaining at emergence. Ether-extractable substances decreased to one half of their original concentration by emergence, to 10% six days later and to 4% 28 days after emergence. Proteins decreased throughout the sampling period to 70% at emergence, 25% nine days later and to a final concentration of 7%.

4. Most of the potassium (69%) and phosphorus (92%) moved out of the cotyledons at about the same rate as the organic reserves. A slight loss in magnesium during germination was followed by an increase which more than made up for the loss (net gain 4%). The calcium content did not change appreciably during germination. In the 28 days after emergence the calcium content of the cotyledons increased 300% over the original concentration.

5. Compared with untreated controls, plants whose cotyledons had been removed at emergence or two days later were reduced in height and showed tendencies toward reduction in seed yield. Cotyledon removal later than four days after emergence had no effect on plant development.

6. No important differences were observed between the two varieties of soybeans in either translocation of food reserves from the cotyledons or in the effect of cotyledon removal on plant development.

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ON THE MASS CULTURE OF ALGAE[1]

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(WITH FOUR FIGURES)

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Of the considerable body of literature variously concerned with the culture of algae there is relatively little work directed toward the particular problem of mass culture. VON WITSCH (**10, 11**) grew Chlorella in vertical glass cylinders of 3 cm. diameter and studied effects of carbon dioxide provision and the composition of the medium on yield and rate of growth. He achieved yields which approached the following as maxima: 5.0 gm. dry weight per liter in 20 days; 6.68 gm. dry weight per liter in 38 days (figures from different experiments). SPOEHR and co-workers (**8**) produced 10.69 kg. of dry Chlorella from 590 cultures grown in 5-gallon carboys under daylight illumination in a greenhouse. The cultures were harvested at 30 to 50 days; the maximum yield achieved was about 4.0 gm. dry weight per liter. No attempt was made to investigate the factors limiting growth rate or yield. KETCHUM, LILLICK, and REDFIELD (**4**) studied growth and optimum yields in cultures illuminated by a jacketed neon tube immersed in eight liters of media contained in a pyrex bottle. Of a number of different unicellular algae examined *Chlorella pyrenoidosa* gave the highest density and daily yield, namely 0.74 gm. per liter maximum density and 0.065 gm. per liter per day maximum growth rate.

If pushed to a stage of practical development, the mass culture of algae will become an engineering problem. Before any such stage is reached, however, the problem is essentially biological. The rates of growth obtained in the work cited above are lower by at least several orders of magnitude than the maximum growth rate of Chlorella of about sevenfold increase per day. The densities of population achieved are far below those obtained with other microörganisms in the fermentation industry. The biological problem is to determine the conditions necessary to obtain maximum density, rate of growth, and efficiency of light utilization.

Sunlight appears to be the only feasible source of illumination for large scale mass culture. The initial experiments have been done, therefore, in a greenhouse under daylight illumination. Since the incident visible radiation per day varies from day to day such experiments must be self-contained, *i.e.*, they must be designed to provide comparative data within any one experiment.

As a starting point we chose to investigate the design of the growth chamber, particularly in regard to the most desirable thickness of the culture ex-

[1] Based on work performed under contract N8onr-78000 with the Office of Naval Research.

posed to illumination. A crude variation of the chamber thickness was achieved by the use of the following four types of chambers which were already available: (1) 5-gallon carboy. (For comparison with the other chambers this is regarded as a short length of a 12-inch vertical tube.) (2) 2-inch diameter vertical tube. (3) 1-inch diameter vertical tube. (4) 6-mm. annulus. (Afforded by use of one of the chambers designed for the continuous culture apparatus used routinely in this laboratory (5). Typical dimensions of the annulus are: 45.0 mm. ID of outer tube and 32.2 mm. OD of inner tube.)

A rough temperature control was obtained by the use of tap water at a temperature of 20 to 23° C. The carboy was cooled by flowing the water over the outside of the bottle. The cooling water was circulated through small glass tubes inserted lengthwise through the 1- and 2-inch chambers and through a jacket surrounding the annular chamber.

The cultures were started by transferring to each of the chambers a portion of a single batch of inoculated medium. An inoculum of *Chlorella pyrenoidosa* was obtained by centrifuging a sample harvested from the continuous-culture apparatus. The medium used was a Knop's solution (with increased nitrate concentration of the following composition: 0.010 M $MgSO_4$, 0.025 M KNO_3, 0.009 M KH_2PO_4, 1×10^{-5} M ferric iron, and 0.5 ml. per liter of the A_5 microelement solution of ARNON (1). Carbon dioxide provision and agitation of the cultures was obtained by aerating with a rapid stream of a carbon dioxide-air mixture (5:95). No attempt was made to maintain pure culture conditions.

Growth was followed in terms of the dry weight of cells produced per unit volume of culture. A measured aliquot from a culture was centrifuged out, washed twice in water, transferred in a small quantity of water to a tared crucible, and dried to constant weight at 110° C.

The results of the experiment are presented in figure 1 as growth curves for the different chambers. Attention is called to three aspects of the data. First, the density of population attained is inversely related to the thickness of suspension exposed to light. Second, the latter portions of the growth curves fit, or at least approximate straight lines. Third, the slopes of these lines (growth rates) also show a definite inverse relationship to the thickness of the suspension exposed to light.

Linear growth curves under controlled laboratory conditions have been explained in terms of the constant rate of provision of some required material. Such a limitation might arise in the present experiments if either the rate of carbon dioxide provision or the rate of light energy absorption becomes limiting. In this and later experiments we have made exhaustive tests to see whether carbon dioxide provision is the limiting factor. A sample of suspension from the 12-inch culture removed at 25 days (fig. 1) and transferred directly to a Warburg vessel contained enough carbon dioxide to support rapid photosynthesis for over an hour. In a later experi-

ment a slow flow of pure carbon dioxide was added to the much greater flow rate of 5% carbon dioxide in one of two parallel cultures in annular chambers. Thus in one culture with an added flow of pure carbon dioxide the high flow of 5% carbon dioxide served to hold down the carbon dioxide concentration in the liquid medium, while in the parallel control culture it served to hold up the carbon dioxide concentration in the medium. There

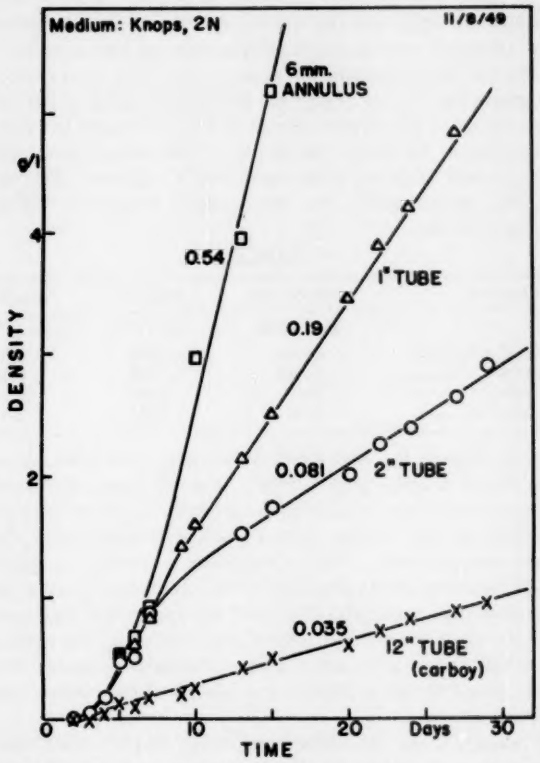

FIG. 1. Growth in cultures of Chlorella under greenhouse illumination in vertical chambers of various thicknesses.

was no significant difference in the rate of growth achieved in the two cultures. Perhaps still more convincing are numerous observations that the pH of a sample changes greatly on aeration in air. A sample taken from an annular culture when the density was 12.12 gm./l. had a pH of 7.95. After several minutes aeration in air the pH had risen to 8.70. Photosynthesis of Chlorella is known to be carbon dioxide-saturated when the surrounding fluid is in equilibrium with as little as 0.1% carbon dioxide (**2**). Rate of

carbon dioxide provision, therefore, is not a limiting factor in the present experiments.

The second possible explanation of the linear growth is that when a culture approaches 100% light absorption it reaches a constant rate of growth determined by the rate of light absorption. The qualitative observation that all the cultures appeared to become opaque supported this possibility. The data of figure 1 on growth rates were reexamined in terms of the total surface exposed to light and the results are summarized in table I. The third column presents the calculated surface area per liter of culture for each chamber. In the fourth column the growth rates have been recalculated in terms of grams per square meter of illuminated surface per day. The growth rate values of the second column in terms of grams per liter per day show a variation of 15 times. In terms of unit area illuminated (fourth column) the growth rates are much more nearly constant. It thus appears that, as a first approximation, the rate of light absorption determines the final linear growth rate.

TABLE I

Chamber	Growth rate	Surface	Growth rate
	gm./l/day	cm.²/l	gm./m²/day
12 inch tube (carboy)	0.035	200	1.8
2 inch tube	0.081	850	0.95
1 inch tube	0.19	1600	1.2
6 mm. annulus	0.54	1800	3.0

It must be pointed out that there is still one other phenomenon which might give rise to a linear growth rate. If a cell population becomes depleted of any essential catalyst, then the growth rate might become limited by the amount of this catalyst even though it is continually divided up among more and more cells. Such a condition is possible in algal cultures since the pH rises and the availability of iron and other trace elements decreases. It should be noted also that even the apparently high growth rate observed in the annular culture is only of the order of 0.1 the maximum rate of growth of Chlorella. It therefore appears desirable to search for possible limitations imposed by the amount or availability of the various components of the nutrient solution.

Growth studies under laboratory conditions had revealed that iron or other microelements may become limiting even though initially provided in adequate amounts. In the mass culture work pure culture conditions were not maintained and the usual chelating agents such as citrate or tartrate were avoided since these would most certainly be attacked by bacterial or mold contaminants. Subsequently the biologically inert chelating agent, ethylene diamine tetra-acetic acid (EDTA), brought to our attention by S. H. Hutner, was tested under laboratory conditions and found useful in removing apparent microelement limitations. The use of EDTA was tested in mass culture conditions under daylight illumination in the greenhouse.

In one of two parallel annular cultures EDTA was added to the Knop's solution with microelements according to the following schedule originally developed for Euglena by S. H. Hutner (personal communication): 30 parts per million Ca, 20 p.p.m. B, 20 p.p.m. Zn, 10 p.p.m. Fe, 4 p.p.m. Mn, 4 p.p.m. Mo, 4 p.p.m. Cu, 1 p.p.m. Co. The other culture received microelements only as previously described. The results of the experiment are shown in figure 2. It is clear that EDTA increases the density to which a population can be carried and that without it some factor, presumably availability of

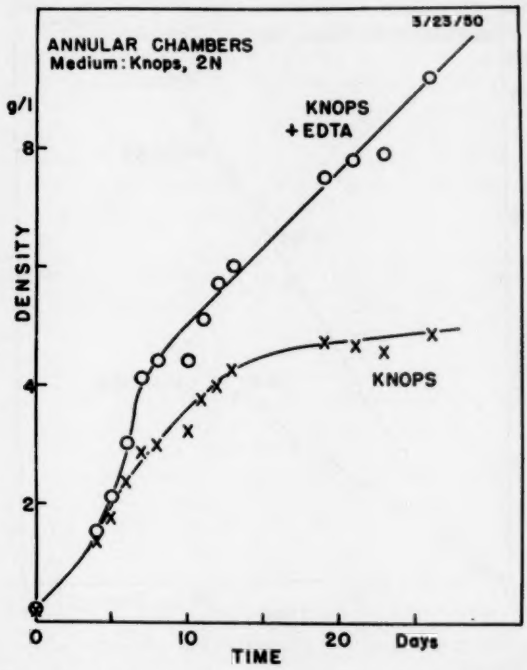

FIG. 2. Effect of the chelating agent EDTA on growth at high densities of population.

microelements, soon becomes severely limiting. Consideration of the function of a chelating agent shows that it will be a very great aid in mass culture work. It is a serious problem to provide adequate amounts of the various microelements, and to maintain their availability, without exceeding the limits of toxicity. The easily reversible chelate complex provides a buffer system that will maintain ionic concentrations at desirable levels throughout the life of a culture.

Following the improvement in growth conditions introduced by the use of EDTA, it appeared desirable to check again upon effects of thickness of

the layer of suspension exposed to illumination. Figure 3 describes an experiment in which an annular chamber was compared to a cylinder of the same outer diameter as the annulus. The annulus had an outer diameter of 45.0 mm. and contained 7.88 cc. for each 1.0 cm. length. The cylinder had an outer diameter of 43.5 mm. and contained 14.5 cc. for each 1.0 cm. of length. The culture medium was Knop's with the nitrate increased to three times the usual level and with EDTA and microelements according to the formula of the preceding experiment. The growth curves of figure 3

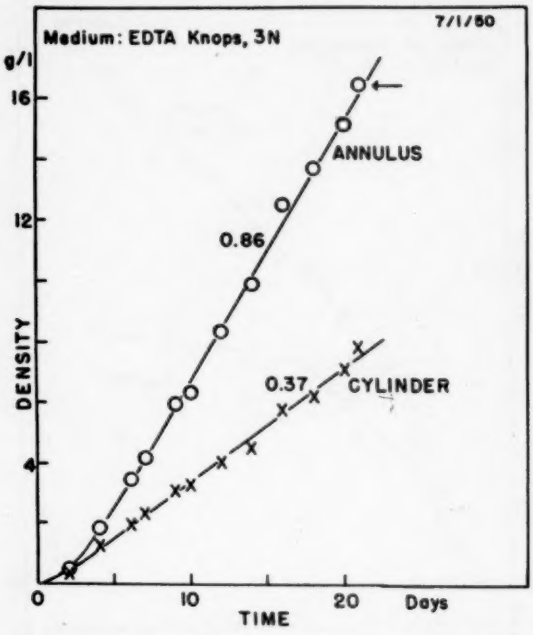

FIG. 3. Comparison of growth in an annulus and cylinder of approximately the same outside diameter.

again indicate a much more rapid rate of growth (figure above each curve) and higher final density in the thinner layer of the annular chamber. The data on growth rates are presented in table II in a treatment similar to that of table I. The growth rate or yield per unit volume of suspension (second column) is over twice as high in the annulus as in the cylinder. In terms of unit surface area (fourth column) the growth rate is only 20% higher in the annular chamber. Again it appears to be a reasonable approximation that the final linear rate of growth is established by the rate of light absorption. Estimates of light absorption in the annular chamber have been made

by inserting a barrier-type photocell up into the inner tube and alternately measuring the light intensity transmitted by the annulus through the suspension or above the suspension (air space). According to these rough-measurements the annular layer in the experiment of figure 3 showed a transmission of 52% at two days and 1.3% at 10 days. During the later portion of the growth curves, roughly corresponding to the period of linear growth, light absorption by the cultures was nearly complete.

The data appear reliable enough to place significance on the 20% higher growth rate per unit surface of the annular chamber. It is instructive to consider the same phenomenon in terms of the geometry of the chambers. In the cylinder the imaginary outer annulus, corresponding to the actual thickness in the annular chamber, contained 49% of the total volume. If this layer had shown as rapid a rate of growth as the annular chamber, the over-all growth rate should have been $0.49 \times 0.86 = 0.42$ plus the contribution of the suspension in the inner core of the cylinder. Comparison to the growth rate observed for the cylinder shows that the inner core was less than useless in its contribution to growth.

TABLE II

Chamber	Growth rate	Surface	Growth rate
	gm./l/day	cm.²/l	gm./m²/day
Annulus	0.86	1800	4.8
Cylinder	0.37	930	4.0

The annular chamber of figure 3 was harvested at 21 days and examined for possible deficiencies in the medium. The suspension was centrifuged and the supernatant further filtered through a sintered glass filter. The filtrate was still slightly turbid; microscopic examination indicated the presence of bacteria and some algal cell debris. The filtrate was dispensed into colorimeter tubes and to duplicate sets there were added the following: (1) no addition, (2) nitrate, (3) phosphate, (4) iron, (5) nitrate plus phosphate plus iron. The tubes were inoculated with some of the centrifuged cells from the culture, placed in an illuminated water bath, aerated with 5% CO_2 and growth followed by periodic measurements of optical density. After a lag period of about 24 hours good growth occurred in all tubes receiving added nitrate. In all other tubes growth was very slow and would have been considered negligible by usual standards. It appears that the nitrate of the original culture had been exhausted.

An approximate calculation of the nitrogen requirement may be made from the nitrogen composition of Chlorella cells. If one selects a value of 8.5% nitrogen as typical (cf. **6**) then it is predictable that the usual concentration of 1.25 gm./l. of KNO_3 in Knop's solution will support only 2.0 gm./l. of Chlorella of this nitrogen content. The annular culture of the experiment of figure 3 developed a maximum population density of 16.5 gm./l. of cells from three times the usual potassium nitrate level of 3.75

gm./l. At the final density attained the cellular nitrogen content probably fell to a value of about 3.1%. It is possible that, as suggested by SPOEHR and MILNER (9), some nitrogen fixation occurs in Chlorella. It seems more likely that, as also observed by Spoehr and Milner, the nitrogen content of the cells varies within wide limits depending upon the conditions of nitrogen supply. Since nitrogen limitations have been shown to limit the rate of

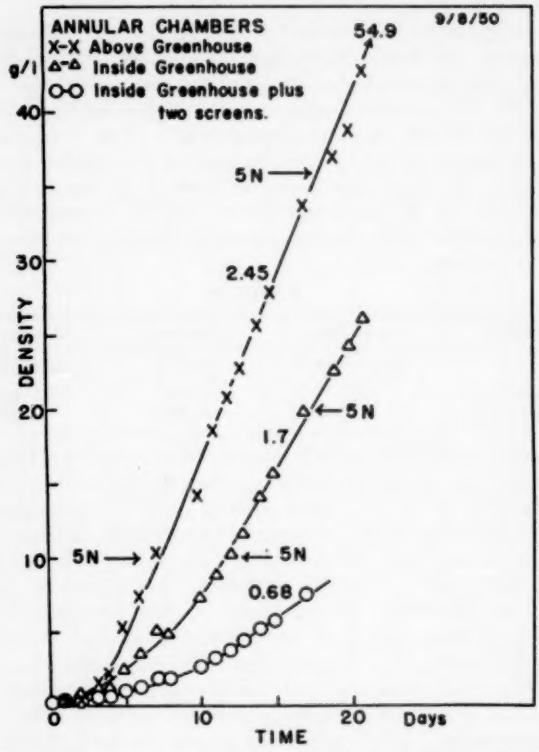

FIG. 4. Growth in cultures maintained under different conditions of illumination.

photosynthesis in Chlorella (VAN HILLE, 3) it is possible that a nitrogen limitation as well as a light limitation establishes the final linear growth rate.

The nitrate concentration may be increased up to about five times the usual value in Knop's solution, *i.e.*, to $5 \times 1.25 = 6.25$ gm./l. of potassium nitrate, without initial inhibitory effects as observed in test tube cultures. Inhibitory effects are observed at a potassium nitrate concentration of 10.0 gm. per l. In order to maintain available nitrogen in cultures growing to a density of greater than 10 gm./l. it will be necessary to make periodic addi-

tions of fixed nitrogen to the culture. The problems related to nitrogen supply are under further investigation.

The experiments described above were performed in a greenhouse with whitewashed glass. The maximum full sunlight intensity was about 1000 foot-candles. Effects of the incident light intensity have been studied by comparing growth in three annular chambers exposed to different conditions of illumination. One chamber was set up in the greenhouse as in the preceding experiments. A parallel chamber was enclosed in two layers of window screen which transmit about 50% of the incident light. A third chamber was elevated well above the roof of the greenhouse so that it received direct sunlight. The culture medium was an EDTA-Knop's solution as used in the preceding experiment but with five times the usual nitrate concentration. Additional nitrate was added as indicated by the arrows in figure 4 which also presents the results of the experiment.

The culture exposed to full sunlight achieved a higher rate of growth (2.45 gm./l./day) and was carried to a higher population density than any algal culture previously reported. At 54.9 gm./l. the culture contained over 5% of its mass as algal substance and about 25% of its volume as algal cells. No real limit upon the maximum achievable density has yet been observed.

Unfortunately, conditions of illumination are not such as to allow quantitative comparison of growth rates and light intensities. Inside the greenhouse the illumination was diffuse with considerable back-lighting. Qualitative comparison, however, shows that higher incident intensities give greater rates of growth.

Discussion

We consider it unlikely that maximum densities or rates of growth of algae have yet been achieved under conditions applicable to mass culture. The related problems of nitrogen provision and pH stabilization have not yet been solved: periodic addition of ammonium nitrate appears indicated but has not yet been tried. There may be an important contribution of flashing light effects caused by turbulence in the culture. There are possible limitations of growth by toxic products of the algae such as the chlorellin of PRATT (7). And finally, there is no certainty that *Chlorella pyrenoidosa* is the most desirable form for purposes of mass culture.

The present work is a first attack on those biological problems of practical importance to the development of algal mass culture. The over-all problem is one which must be pursued as a series of successive approximations toward maximum density, rate of growth, and efficiency of light utilization. The path of future development is now defined by the following considerations:

1. The mass culture of a hardy and rapidly growing form such as *Chlorella pyrenoidosa* does not require pure culture conditions. Bacterial contaminants have appeared in small amounts in all of our cultures but there has been no evidence of any inhibiting effects.

2. Maximum density, rate of growth, and efficiency of light utilization require the use of thin layers of suspension exposed to light. High density of population is of critical importance since it permits more economical harvesting of the final crop.

3. The use of a biologically inert chelating agent such as ethylene diamine tetra-acetic acid solves the problem of providing trace elements and maintaining their availability.

4. Because of the high nitrogen content of Chlorella, special provision of nitrogen in the medium must be made in order to achieve high density cultures.

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THE POSITION OF C^{14} IN SUNFLOWER LEAF METABOLITES AFTER EXPOSURE OF LEAVES TO SHORT PERIOD PHOTOSYNTHESIS AND DARKNESS IN AN ATMOSPHERE OF $C^{14}O_2$[1]

MARTIN GIBBS

Received January 6, 1951

The rate at which assimilated carbon dioxide is distributed in the carbon atoms of plant sugars and other plant metabolites should provide indication of the interrelationships between the sugars and these substances after exposure of the plant to carbon dioxide in the light and in the dark.

WOOD and BURR (**9**) were the first to determine the distribution of isotopic carbon in the carbohydrates of a bean plant allowed to photosynthesize in $C^{13}O_2$. They found that sucrose had a higher C^{13} isotope content than the hexoses, dextrin or starch. They also noted that there was a difference in the C^{13} content in the various carbon atoms of the sugars. CALVIN and BENSON (**2**) have reported that during short period photosynthesis in $C^{14}O_2$ sucrose was the only non-phosphorylated carbohydrate-containing label. The specific activity of carbon atoms 3,4 of the hydrolyzed sucrose was higher than carbon atoms 2,5 and 1,6. In some experiments, the specific activity of carbon atoms 2,5 was equal to carbon atoms 1,6 while in others they were unequal. Malic acid was predominantly carboxyl labeled. The distribution of label in alanine was somewhat similar to that of hexose. The carboxyl carbon having the highest specific activity, the beta carbon the least.

GIBBS (**3**) reported that with a one-hour period of photosynthesis by barley plant in $C^{14}O_2$, the distribution of label in the hexoses was the reverse of that published by the California workers (**2**), *i.e.*, carbons 1,6 had the highest specific activity. Recently VITTORIO, KROTKOV, and REED (**8**) have shown that the discrepancy between the result in the two studies was due to a dilution of carbon atoms 3,4 by $C^{12}O_2$ after the hexose was uniformly labeled.

VARNER and BURRELL (**7**) found the same distribution of label within the carbon atoms of the soluble hexoses as in the glucose from starch isolated from *Bryophyllum calycinum* leaves which had photosynthesized for 30 minutes in $C^{14}O_2$, carbon atoms 3,4 having the highest specific activity. Malic acid isolated from Bryophyllum leaves which had been exposed to $C^{14}O_2$ in the dark for 2.5 hours was predominantly carboxyl labeled.

The present work is concerned with the distribution of C^{14} in the glucose, fructose, sucrose, dextrin, starch, alanine, and malic acid of sunflower leaves

[1] Research carried out at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

after a short period of photosynthesis in $C^{14}O_2$. The distribution of isotope in the same compounds was also determined after exposure of sunflower leaves to isotopic carbon dioxide in the dark. The sunflower leaf was selected since it synthesizes carbohydrate in light and dark at a suitable rate.

Methods

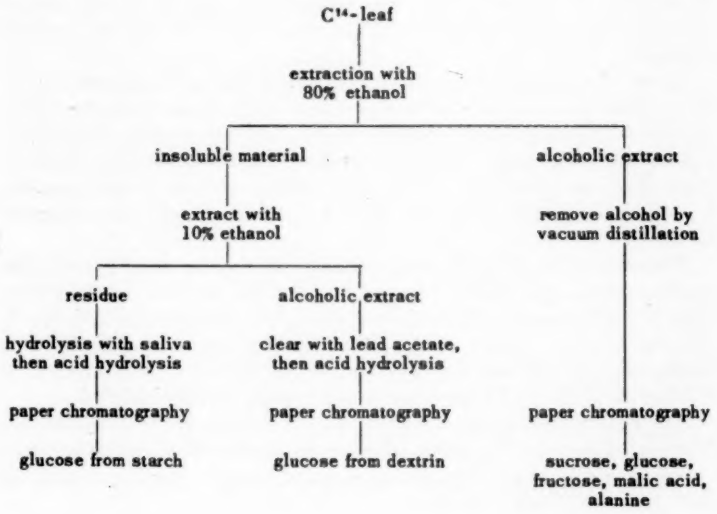
Exposure of leaves to isotopic carbon dioxide

A detached leaf of a sunflower plant was allowed to photosynthesize in air for 15 minutes in a chamber similar to that of Aronoff *et al.* (1). The leaf was then exposed to carbon dioxide containing 100 microcuries of C^{14} released from 500 mg. of barium carbonate for one and one-half, two or four minutes. The temperature was 20° C and the illumination was equivalent to 10,000 foot-candles. After exposure, the chamber was swept three minutes with CO_2-free air. The leaves were then immersed in boiling 80% alcohol for 20 minutes.

The leaves used in the dark exposure experiments were taken from plants previously kept in the dark for 40 hours. The petioles of the detached leaves were placed in a few ml. of a 7% glucose solution, then exposed at 25° C to $C^{14}O_2$ in the dark for 16 and 27 hours. The leaves were then immersed in boiling 80% alcohol for 20 minutes.

Isolation of C^{14}-labeled compounds

The isolation of the C^{14} metabolites is summarized in the following flow sheet:



The initial alcoholic extract was evaporated under vacuum at room temperature to approximately 2 ml. About 20 microliters of this solution was subjected to 2-dimensional paper chromatography. Phenol and a mixture of butyl alcohol and propionic acid were the solvents. To isolate the glucose from fructose or sucrose, the sucrose spot was cut out of the paper chromatogram, the sucrose eluted with water, hydrolyzed with invertase, and rechromatographed on paper with phenol as solvent (**6**). The alanine and malic acid were obtained for degradation by cutting out the appropriate spots from the 2-dimensional chromatogram and eluting the compounds with warm water.

The material remaining after the 80% ethanol extraction was placed in a 50-ml. round bottom centrifuge tube and dried overnight in a vacuum oven. After drying, a small amount of sharp sand was added and the sample frozen by pouring about 25 ml. of liquid nitrogen into the tube. The frozen leaf was ground to a fine powder with a thick stirring rod. The ground tissue was extracted four times with cold 10% alcohol. The 10%

TABLE I

DEGRADATION OF SYNTHETIC LACTIC ACID ACTIVITY IN MILLIMICROCURIES (1×10^{-3} MICROCURIES) PER MG. OF CARBON

	Carbon atom		
	COOH	CHOH	CH_3
Lactic acid-1-C^{14}	273.0	0.0	0.0
Lactic acid-2-C^{14}	2.3	209.0	0.0
Lactic acid-3-C^{14}	0.0	0.62	25.8

alcohol extract which contained the dextrin, was cleared with lead acetate and hydrolyzed with 1 *N* sulphuric acid. The acid was removed by addition of solid barium carbonate. The glucose from the dextrin was isolated by paper chromatography. The residue remaining after the 10% alcohol extraction which contained the starch was dried, hydrolyzed with saliva, and then treated in the same manner as the 10% alcohol extract.

Degradation procedures

The sugars were degraded by the method of WOOD, LIFSON, and LORBER (**8**). The reactions are:

1. $\overset{1}{CHO}-\overset{2}{CHOH}-\overset{3}{CHOH}-\overset{4}{CHOH}-\overset{5}{CHOH}-\overset{6}{CH_2OH} \xrightarrow{\text{Lactobacillus casei}} \overset{1,6}{CH_3}-\overset{2,5}{CHOH}-\overset{3,4}{COOH}$

2. $CH_3-CHOH-COOH \xrightarrow{KMnO_4} \overset{1,6}{CH_3}-\overset{2,5}{CHO} + \overset{3,4}{CO_2}$

3. $CH_3-CHO \xrightarrow{NaOI} \overset{1,6}{CHI_3} + \overset{2,5}{HCOONa}$

Test of the fermentation (reaction 1) has been carried out (**4**). The iodoform resulting from reaction 3 is oxidized to CO_2 by chromic acid and the formate to CO_2 by mercuric oxide. Table I presents a test of reactions 2 and 3.

Alanine was degraded by the following reactions:

1. $\overset{3}{CH_3}-\overset{2}{CH(NH_2)}-\overset{1}{COOH} \xrightarrow{\text{ninhydrin}} \overset{3}{CH_3}-\overset{2}{CHO} + \overset{1}{CO_2} + NH_3$

2. $CH_3-CHO \xrightarrow{NaOI} \overset{3}{CHI_3} + \overset{2}{HCOONa}$

Table III represents a test of the alanine degradation procedure.

The malic acid was degraded by the following reactions (**11**):

1. $\overset{4}{HOOC}-\overset{3}{CH_2}-\overset{2}{CHOH}-\overset{1}{COOH} \xrightarrow{KMnO_4} \overset{3}{CH_3}-\overset{2}{CHO} + \overset{1,4}{CO_2}$

2. $CH_3-CHO \xrightarrow{NaOI} \overset{3}{CHI_3} + \overset{2}{HCOONa}$

The iodoform and formate of the alanine and malic acid degradation were treated as described under sugar degradation.

TABLE II

DEGRADATION OF SYNTHETIC ALANINE-2-C^{14}

Carbon atom	mμc./mg. carbon
COOH	0.12
$CH(NH_2)$	823.0
CH_3	0.06

The C^{14} was determined as $BaCO_3$ on a sintered disk and counted with a methane flow proportional counter. The techniques of counting C^{14} and the method of oxidizing carbon compounds for total activity used in this laboratory have been described by STEELE and SFORTUNATO (**5**).

Results and discussion

Of the various carbohydrates, the free monosaccharides (glucose and fructose) contained no label. This fact is in agreement with the mechanism for photosynthesis proposed for Chlorella, barley, and Scenedesmus (**2**).

The results of the degradation of the compounds isolated from sunflower leaves exposed to strong light in the presence of $C^{14}O_2$ for short periods of time are shown in table III. The figures represent percentages of the total C^{14} in the various carbon atoms. Carbon atoms 3,4 had the highest activity in all the sugars. In the 1.5-minute and 4-minute exposures, the specific activity of carbon atoms 2,5 was practically equal to 1,6. In the 2-minute

exposure, the specific activity of position 2,5 of sucrose was greater than 1,6. Unequal (**2, 3, 4, 5**) and equal (**2**) distribution of activity in 2,5 and 1,6 has been reported. The factors leading to this peculiar distribution of label are obscure. With opposite leaves from the same plant, an equal distribution of C^{14} between 2,5 and 1,6 was found with one, an unequal distribution with the other. It seems possible that the hypothetical C_2 fragment might be formed by two pathways, one of which passes through a symmetrical molecule. The degree of labeling would then depend on the rates of the two mechanisms. The glucose and fructose derived from sucrose during the 1.5-

TABLE III

DEGRADATION OF PHOTOSYNTHETIC PRODUCTS. FIGURES ARE PERCENTAGES OF C^{14} IN VARIOUS CARBON ATOMS.

Compound	1.5 min.	2 min.	4 min.
Sucrose			
Fructose			
3,4	48	52	37
2,5	26	31	33
1,6	25	17	30
Glucose			
3,4	48	51	
2,5	25	31	
1,6	27	18	
Dextrin			
3,4	36		37
2,5	33		35
1,6	31		28
Starch			
3,4	72		61
2,5	14		17
1,6	14		22
Alanine			
COOH	47		41
$CHNH_2$	28		29
CH_3	25		30
Malic Acid			
COOH	76		76
CHOH	11		12
CH_2	13		12

and 4-minute periods of photosynthesis had a similar distribution of label which would indicate a common source.

The dextrin achieved uniform labeling the fastest of the carbohydrates. In spite of this, the starch was synthesized slowly from the newly formed carbohydrates. If the plant had been starved, the label in the starch might have been equal to the dextrin and sucrose.

The distribution of label in the alanine was the same as in sucrose. The carboxyl carbon predominated while the alpha carbon was practically equal to the beta carbon. With increasing exposure time, the distribution became more uniform. The malic acid was predominantly carboxyl labeled and did

not change with time. The specific activity of the alpha carbon was equal to the beta carbon. The 2-dimensional radiograms of the alcoholic extract which contained the alanine and malic acid were similar to those of BENSON and CALVIN (**2**). The alanine showed a dark spot while the malic acid showed a very faint spot. This result has been interpreted by these workers to indicate that strong light inhibits the formation of compounds of the tricarboxylic acid cycle. The degradation data would tend to substantiate this interpretation.

The results of the degradation of compounds isolated from sunflower leaves which had been exposed in the dark to an atmosphere of $C^{14}O_2$ for 16 and 27 hours are shown in table IV. The figures represent percentages

TABLE IV

DEGRADATION OF DARK FIXATION METABOLITES. FIGURES REPRESENT PERCENTAGES OF C^{14} IN VARIOUS CARBON ATOMS.

Compound degraded	16 hours	27 hours
Sucrose		
3,4	90	87
2,5	5	6
1,6	5	7
Dextrin		
3,4	90	
2,5	5	
1,6	5	
Starch		
3,4	84	96
2,5	8	1
1,6	8	3
Alanine		
COOH	98	100
$CH(NH_2)$	0	0
CH_3	2	0
Malic Acid		
COOH	92	
CHOH	4	
CH_3	4	

of C^{14} in the various carbon atoms. The dark exposure experiments were carried out to determine whether carbon dioxide fixation by a leaf in the dark was similar to fixation of carbon dioxide by non-photosynthetic organisms. As in the photosynthesis experiments, the monosaccharides contained no label. It is evident from table IV that the carbohydrates contained practically all their C^{14} in positions 3,4 while alanine and malic acid were carboxyl labeled. The presence of label in positions 1,2,5,6 of sugar was partially caused by the *L. casei* fermentation during degradation (**4**).

Mammalian tissues fix carbon dioxide in the 3,4 position of glucose (**10**) and lower organisms fix CO_2 in the carboxyl groups of organic acids. Since a similar distribution of label occurred in the sunflower metabolites, it would indicate that the carbon dioxide entered the plant metabolites by a Wood-

Werkman or similar reaction and in the case of the sugars followed by the reverse of glycolysis.

The distribution of label in the sunflower metabolites after exposure of a leaf to light in the presence of $C^{14}O_2$ in contrast to its distribution in the dark indicate that light is necessary to initiate the cycle which synthesizes the C_2 acceptor.

Summary

Detached sunflower leaves were allowed to photosynthesize in strong light for a short period in an atmosphere of $C^{14}O_2$, after which the carbohydrates (sucrose, glucose, fructose, dextrin, and starch), alanine, and malic acid were isolated and the location of the C^{14} determined. The same compounds were isolated and degraded from leaves which had been exposed to $C^{14}O_2$ in darkness.

1. In the photosynthesis experiments: (a) The monosaccharides contained no label. (b) Dextrin approached uniform distribution of carbon more rapidly than the other substances. (c) The labelling of alanine was similar to that of sucrose. (d) Malic acid remained predominantly carboxyl labeled during all exposures.

2. In the dark experiments: (a) Of the carbohydrates, glucose, fructose, and sucrose, only the sucrose contained label. When the sucrose was inverted and degraded, the invert sugar was labeled only in carbon atoms 3 and 4. (b) The alanine and malic acid were labeled only in the carboxyl carbon.

3. The data indicate that before label can occur in positions other than carbon atoms 3 and 4 of sugar and the carboxyl carbon of amino acids and organic acids, light is necessary to initiate the cycle which synthesizes the C_2 acceptor.

The author wishes to thank Dr. B. M. Tolbert, Radiation Laboratory, University of California, for the chemically synthesized C^{14}-lactic acid and C^{14}-alanine. He also wishes to thank Miss Ruth Gastel and Mrs. Frances A. Bennett for their able assistance. He also expresses appreciation to Dr. Robert Steele of this laboratory for frequent valuable suggestions made during the course of this work.

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THE EFFECT OF PETIOLE TEMPERATURE ON THE TRANSLOCATION OF CARBOHYDRATES FROM BEAN LEAVES[1]

C. A. Swanson and R. H. Böhning

(WITH FOUR FIGURES)

Received February 16, 1951

The present controversial status of the problem of transport rate as related to temperature is briefly summarized in a recent paper by Went and Hull (**8**). As pointed out by these authors, the reported Q_{10} values for translocation range from less than 1 (**7, 8**), to essentially 1 (**5**), to values greater than 1 (**3, 4**).

The effects of temperature are multiple and greatly complicate an analysis of this problem. It is to be expected, though little data exist for its substantiation, that the effect of temperature on rate of conduction of carbohydrate materials through petioles, will be influenced by temperature effects on numerous other processes occurring in various parts of the plant, such as respiration and assimilation, and the effect these processes have on concentration gradients between regions of export and import. Studies, therefore, in which the entire plant is subjected to different temperatures (**4**), although contributing much data of value, do not permit a clear analysis of the temperature effect on the process of translocation *per se*. In the present experiments, therefore, only the petiole temperature was varied, the rest of the plant in all treatments being maintained at a temperature of $20 \pm 1°$ C.

Materials and methods

The report by Weintraub and Brown (**6**) that stem elongation of *Phaseolus vulgaris* var. Black Valentine was directly proportional, within the range of 0 to .75 *M*, to the concentration of sugar supplied to the leaves suggested that this relationship could be used as a basis of approach for studying the effect of temperature on translocation of carbohydrates. If temperature has an effect on the rate of movement of sugar through the petiole then the amount of sugar reaching the growing tip will vary with temperature and the rate of elongation should vary accordingly. Stem elongation may then be used as an index to the effect of temperature on translocation of sugar. The basic features of the method, therefore, consisted of jacketing the petiole of one primary leaf, immersing the blade in sugar solution and measuring the subsequent elongation of the plant occurring in the dark.

Seeds of *Phaseolus vulgaris*, variety Burpee's Stringless Greenpod (in

[1] Papers from the Department of Botany and Plant Pathology, The Ohio State University, Columbus 10, Ohio. Number 529.

earlier experiments) or Black Valentine (in later experiments), were planted in sand and allowed to germinate under greenhouse conditions. As soon as the hypocotyls had straightened (6 to 8 days), selected seedlings were transplanted to soil in 2½-inch pots, one per pot, and maintained in the greenhouse until the plants developed to a size suitable for use in the experiment. The plants were then transferred to a dark room for a period of 24 to 48 hours to deplete the carbohydrate reserves of the plants. Following the dark period the plants were transferred to specially constructed temperature units. Each of these units (referred to hereafter as "plant units") held 10 plants, and eight such units were employed in each experiment. Each experiment, therefore, comprised a total of 80 plants. During each run the plant units were kept in a dark cabinet thermostatted at 20 ± 1° C.

The box-type temperature jackets (A, fig. 1) were constructed from ¼-inch tempered masonite. Four of these units were provided with electric heating elements, consisting of four lengths of no. 18 gauge nichrome wire in both the top and bottom of each box, and joined serially. With proper voltage regulation, it was possible to maintain the temperature within these boxes at any desired level above the cabinet temperature, within a range of ± 1° C.

In addition to the four electrically heated boxes, two boxes were refrigerated by circulating cold water through brass pipes mounted inside the boxes. The cold water was provided by a refrigerated reservoir set up outside of the cabinet and cooled by a ⅓ H.P. cooling unit. The bath was thermostatted at 1.0 ± 0.5° C, but in warm weather, this low temperature setting exceeded the capacity of the refrigerating unit and the effective operating temperature was about 4° C and occasionally even higher. A centrifugal pump with submerged pump casing was used to circulate the water through the system. Rectangular brass tubing 1 inch wide × ½ inch deep × 32 inches long was used as the cooling element in one box, and ⅜-inch diameter brass tubing in the other. Although connected in series with each other, the difference in heat exchange area of these two units was sufficient to maintain a 5 to 7° C differential between the two boxes. The actual operating temperature of these boxes depended on the degree of cooling attained in the reservoir, the temperature of the one box varying from 5 to 10° C, and the other box from 10.5 to 16° C for different experiments. During any given experiment, however, the temperature for each box varied only by ± 1.5° C.

The remaining two boxes were not equipped with heating or cooling elements and therefore provided the same temperature as the cabinet, *i.e.*, 20 ± 1° C.

As illustrated in figure 1, the pots (E) were packed in vermiculite (F). This procedure, in addition to facilitating watering, permitted simple adjustments during setting up of the experiments for the varying heights of hypocotyl, by the simple expedient of burying the pots to greater or lesser depths in the vermiculite.

After the plants had been mounted in the plant units, the length of the

epicotyl, from primary leaf node to the tip of the youngest bud (referred to hereafter as the stem measurement), and the length of first trifoliate leaf, from its axil to the tip of its terminal leaflet (referred to hereafter as the leaf measurement), were taken to the closest millimeter. The blade of the treated leaf (B) was then rolled and immersed in nutrient (sucrose) solution (C), the blade of the opposite leaf (D) removed, the plant units installed

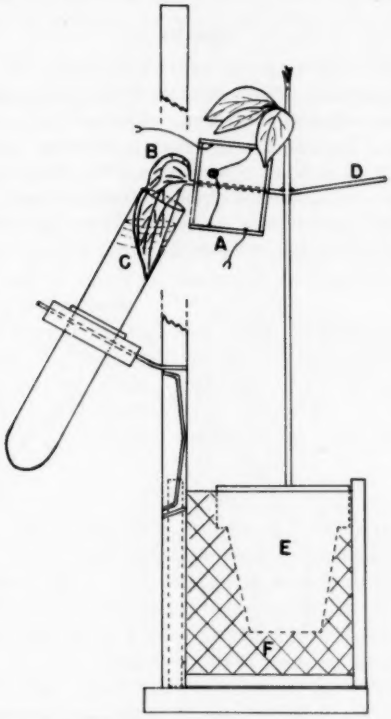

FIG. 1. End view of apparatus. A, unit for controlling petiole temperature; B, blade of primary leaf rolled and inserted into test tube containing nutrient solution C; D, petiole of opposite primary leaf from which blade has been removed; E, 2¼-inch pots embedded in vermiculite, F.

in the thermostatted dark cabinet, and connections made to the heating and cooling circuits. Since the plants had been previously depleted, at least partially, of readily available carbohydrates, continued growth of the stems and leaves would be expected to be mainly limited by the rate of supply of sucrose to the growing parts as influenced by the temperature of the conducting bridge, the petiole.

The nutrient solution used in these experiments was 0.75 *M* sucrose, 0.025% sulfanilamide, and 0.2% Tween-20, in distilled water. In each plant unit, seven plants were supplied with this solution, and three plants, serving as controls, with the same solution minus sucrose.

Final measurements of growth (stem and leaf elongation) were made after four to five days in the dark. In three experiments, intermediate measurements were taken about midway of the experiment (65 to 75 hours).

Results

Six experiments employing this indirect technique for measuring translocation were carried out. Plants were used which had grown to the proper experimental stage under winter, spring, and essentially summer conditions in the greenhouse. Despite this diversity in previous history, substantial agreement was obtained in all experiments. No exception occurred in the basic pattern effect, although the absolute growth values varied considerably for the different experiments, and for the two varieties. Results from one experiment are presented in figure 2.

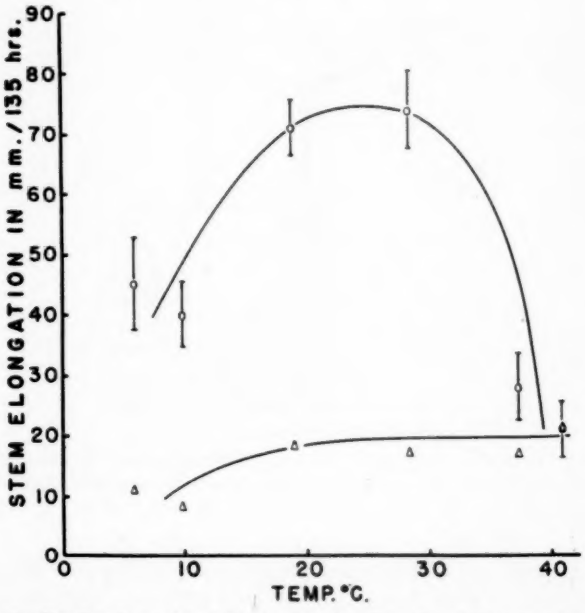

FIG. 2. Results of experiment 5A showing the effect of petiole temperature on the elongation of the stem at 19° C in the dark. Sugar supplied plants ○——○; check plants △——△. Vertical lines show standard error, based on 14 plants at 19° and 28.5° and seven plants at the other temperatures. Difference between 6° and 19° points significant at 2% level; between 28.5° and 37.5°, at the 1% level.

It can be observed from this graph that the maximum rate of transport, as measured in terms of maximum elongation of stem, occurred at petiole temperatures around 20 to 30° C. At temperatures both above and below this range, elongation rate and presumably, therefore, transport rate were considerably reduced.

The difference between means at the optimal and above-optimal petiole temperatures used in these experiments was characteristically very significant. In certain experiments, however, the difference between the optimal and sub-optimal petiole temperatures was significant only at the 5 to 6% level.

Growth of the check plants as related to petiole temperature followed essentially the pattern indicated in figure 2 (curve Δ——Δ). In a few experiments a more distinct type of optimum curve was indicated, somewhat corresponding to the curve for the sugar-supplied plants. Although the sample size of check plants was insufficient to establish statistical significance for the differences in elongation at the different treatments, there is the suggestion that petiole temperature influenced the transport of the naturally occurring foods in much the same way as it influenced the transport of externally supplied sucrose.

It should be emphasized that the above inferences relating transport rates to temperature are based on experiments in which the time factor was of considerable duration (65 to 135 hours). It is recognized that plants growing in the field are subjected to a variable temperature program, and the inferences drawn from the above studies may not apply to field conditions.

Discussion

Figure 3 is a comparison of the results of HEWITT and CURTIS' experiments (**4**), which cover essentially the same temperature range, with those of the present investigation. In comparing these results, it is essential to recall that Hewitt and Curtis' data were obtained by measurement of dry weight losses of plants which were subjected in their entirety to the specified temperatures. In the present experiments, only the petiole temperature of the sugar-fed leaf was varied, the rate of conduction across this temperature bridge being measured in terms of elongation rate of stems and leaves in the sugar-limited plants. Furthermore the time-factor was very different in the two experiments: 14 hours vs. 65 to 135 hours, respectively.

Despite these differences in experimental approach, the results are in remarkable agreement in suggesting an optimal translocation temperature in the 20 to 30° C range. At the extremes of the range, however, certain divergencies are apparent: at the lower temperature levels Hewitt and Curtis' data show a greater depressing effect of temperature than those in the present experiments; at the higher temperature levels the converse relationship obtains. The latter divergency is perhaps largely to be explained in terms of the greater injury effect on protoplasm in the present experiment

as a result of the longer time factor used. The injurious effects of only moderately high temperatures (30 to 40° C) are well brought out in figure 4, which shows a progressive decrease in elongation rate with time at these temperature levels. On the other hand, elongation rate increased with time at the low temperature levels, suggesting a physiological readjustment of the transport mechanism at these low temperatures. This latter phenomenon was noted much earlier, and also more strikingly, in the experiments of

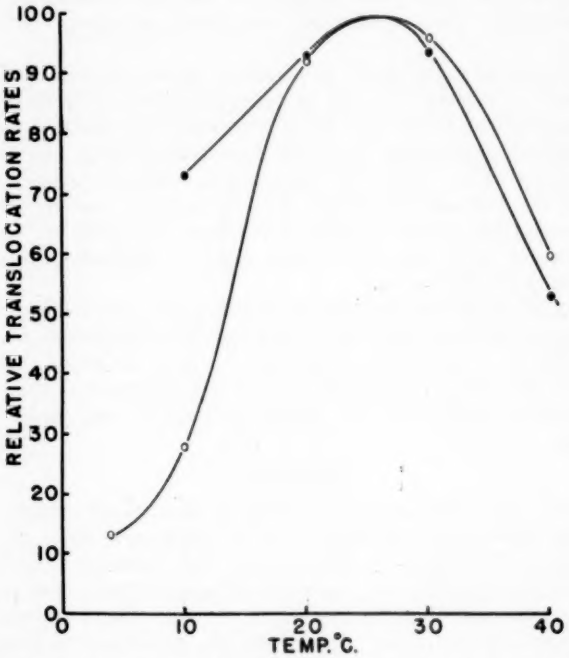

FIG. 3. Comparison of data from Hewitt and Curtis ○——○ (4, redrawn from fig. 8), obtained from dry weight changes with data from the present experiments ●——●, obtained by measurements of growth rates as an index to translocation rates. Latter curve based on the averaged data from six experiments, each calculated relative to 100.

CHILD and BELLAMY (1). These investigators noted considerable diminution in the retarding effect of low temperatures on transport rate in stems of bean and strawberry plants and Bryophyllum petioles after two to three days exposure. This observation, as well as the additive effect of low temperatures operating on all processes throughout the entire plant, may help to explain the divergence in the present results with those of Hewitt and Curtis at the lower temperature levels. CRAFTS (2) observed that cooling

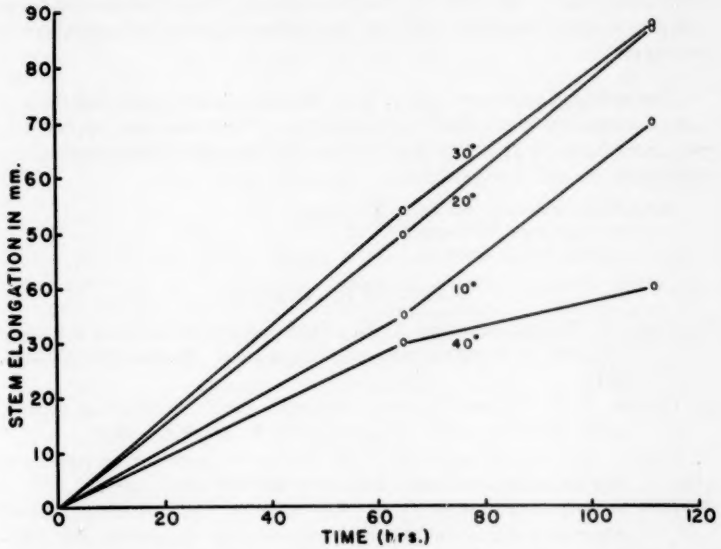

FIG. 4. Stem elongation (Expt. 6) of sugar-supplied bean plants (var. Black Valentine), in dark, as a function of time and petiole temperatures of 10°, 20°, 30°, and 40° C. Points taken from smoothed curves. Similar results obtained for both stem and leaf elongation in all experiments.

entire cucumber plants to 17° C reduced the rate of exudation from the stems considerably more than local chilling of the stems alone to 1 to 4° C.

Summary

The effect of temperature, in the range from 5 to 40° C, on the rate of carbohydrate transport in bean plants, was investigated. An indirect method was used: rate of transport was measured in terms of rate of elongation of the stem and first trifoliate leaf. By means of specially devised temperature jackets, only the temperature of the petiole was varied, the other parts of the plants being maintained at 20 ± 1° C for all treatments. Growth during the experimental period (65 to 135 hours duration for the different experiments) occurred in complete darkness, sucrose being supplied to the plants by immersion of the blade of the temperature-treated leaf in 0.75 molar sucrose solution.

Maximum sucrose transport (maximum elongation of stem and leaf) occurred at petiole temperatures in the range of 20 to 30° C. At petiole temperatures of 5 to 7.5° C, and 40 to 42° C, rate of transport was reduced as much as 50% and 100% respectively, compared with controls.

At 20° C petiole temperature, the rate of elongation of either stem or leaf remained essentially constant throughout the experimental period (135

hours maximum). At lower temperatures, the retarding effect on translocation progressively decreased with time; at higher temperatures, progressively increased.

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TEMPERATURE-INDUCED SAP FLOW IN EXCISED STEMS OF ACER[1]

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(WITH ONE FIGURE)

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Introduction

The flow of sap from openings in the stems of Acer and some other woody genera has for many years received considerable attention and study. In maple, the flow is induced by temperature changes and a review of the literature indicates that practically all of the experimental work has been conducted in the field. It seemed desirable therefore to investigate some aspects of the flow mechanism in the laboratory where temperatures could be controlled.

Literature

The early observations on exudations from plant parts induced in various ways were comprehensively treated by PFEFFER (**21**). Recently KRAMER (**16**) and CRAFTS *et al.* (**10**) have published monographs relating to the subject.

Several different mechanisms have been proposed to explain maple sap flow. One of these is the thermal expansion of gases in the xylem which results in a pressure and a flow of sap if there is an opening in the xylem cylinder. CLARK (**8, 9**) made the first extensive observations on air temperatures, sap pressures, and sugar concentrations during maple sap flows. The simultaneous changes and apparent correlations between air temperature and sap pressures influenced Clark in considering flow to be the result of gas expansion. During the comprehensive and important investigations of JONES *et al.* (**14**), the question of gas expansion as a causative agent in maple sap flow was again considered and for the first time data on the gas content of the vessels were made available. These authors concluded that gas expansion alone was not sufficient to cause a flow. Shortly afterwards, WIEGAND (**24**) reviewed the Vermont data and added some of his own observations. He also concluded that neither gas expansion, water expansion, wood expansion, nor combinations of these could account for the flows observed. The valuable contribution of STEVENS and EGGERT (**22**) demonstrating sap flow from excised stems of red maple is interpreted in terms of another physical explanation: changes in volume may be associated with the formation of ice crystals in the xylem and phloem.

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JOHNSON (**13**) has concluded from his studies of maple sap flow that the most probable effect of alternately high and low temperatures on flow is the effect on the oxygen content of the sap. He maintains that with high temperatures oxygen is less soluble and thus more is available for vital activities such as respiration. Therefore the period of increased respiration coincides with the period of greatest flow.

Many investigators have attributed the flow of sap to an osmotic effect. CLARK (**8, 9**) considered it but did not favor an osmotic explanation. On the contrary, JONES *et al.* (**14, 15**) conclude that the flow is the result of the "bleeding" of living cells and is essentially an osmotic mechanism. WIEGAND (**24**) is of the same opinion and he pictured in some detail the effect of a thermal gradient along a vascular ray which resulted in different temperatures at the two ends of a ray cell. This temperature unbalance would cause an osmotic unbalance and result in the movement of water or of a solution from the unwarmed to the warmed ray cells. He further postulated that the solution would either be secreted or moved by an osmotic gradient into the vessels.

Other investigators have considered the biochemical aspects of sap flow. The enzyme systems responsible for the hydrolysis of starch to sugars have been investigated by BOIS and NADEAU (**2, 3**) and BOIS and CHUBB (**1**) who postulate a starch to sucrose system. MEEUSE (**19**) also investigated the amylolytic properties of maple and birch sap and questioned the starch to sucrose system of BOIS *et al.* (**1, 2, 3**). They found amylase activity, but the concentration of the enzyme was very low. By incubating soluble starch with saps, maltose was produced as well as glucose. Seasonal changes in the carbohydrate content of the maple have been studied in great detail by JONES and BRADLEE (**15**).

The important problem of the composition of the vessel sap in the spring and the changes that occur in the composition of the sap at this time of year have been carefully investigated by BURSTRÖM and KRÖGH (**6, 7**) and BURSTRÖM (**4, 5**). These studies clearly show the rapid and profound changes occurring at the time of bud opening.

Although it has long been known that a flow of maple sap may be obtained from excised stems, recent work by STEVENS and EGGERT (**22**) demonstrates that excised stems absorb during periods of cooling between flows. Presumably in intact plants most of the absorption from roots also occurs on cooling. The mechanisms responsible for the accumulation of water and solutes in the vessels of roots are therefore of great significance. The discussions of EATON (**12**), CRAFTS and BROYER (**11**), LUNDGÅRDH (**17, 18**), VAN OVERBEEK (**23**), and NIE *et al.* (**20**) are pertinent.

Materials and methods

For many years casual observations have indicated that a flow of sap may be obtained from maple logs detached from the roots. STEVENS and EGGERT (**22**) have clearly demonstrated that such stems, if supplied with water, flow in what is apparently a normal manner.

There may be more than one kind of stimulus for the flow mechanism but, so far as is known at present, only a rise in temperature will produce a flow. It seemed desirable therefore, to have a controlled temperature apparatus in which the flow response of maple stems could be studied. An insulated chamber was constructed and supplied with refrigeration and heat. A fan with a 250 cubic feet per minute capacity circulated the air through the chamber and controlled the air temperature to $\pm$ 1° C. Temperatures in the experimental chamber and in the xylem of the stems were recorded by a recording potentiometer using copper constantan thermocouples. The thermocouple points measuring xylem temperatures were inserted obliquely two and a half to three inches in the tissues, approximately in the center of the stem, to reduce to a minimum the effect of thermal conduction along the wires.

The first experiments were on large stems 25 feet long and four to eight inches in diameter which weighed 45 to 55 kg. The cut ends were enclosed in metal caps with rubber gaskets and the flow from tap holes in the stems collected outside the box by means of a plastic tubing.

This procedure proved much too awkward and the apparatus and technique were redesigned to be used with much smaller stems. The stems used for the data reported here were four and a half feet long, 2 to 3 cm. in diameter, and weighed from 0.3 to 0.7 kg. These were from individual plants of *Acer saccharum* collected while frozen. They were either used immediately or collected in quantity in March and stored at – 5° C. Such stored material was still usable after as long as four months under refrigeration. In nature, maple stems collected in Vermont show active flow from about November 15 to leaf emergence. Thus with the aid of frozen material in storage, experiments on flow could be conducted for nine months of the year.

The stems, which extended through both walls of the experimental box, had their ends enclosed with acrylic resin caps. The cap on one end was filled with distilled water and sealed off with glass stopcocks. The flow data were recorded from the other (basal) end of the stem with the apparatus illustrated in figure 1. The cap C and enclosed stem D was attached to a 10-ml. pipette A. At the start of an experiment the distilled water in the cap C was adjusted to form a meniscus near the bottom of the pipette and the stopcock E was closed. After warming the stem, the change in volume was measured in the pipette. In certain experiments solutions were perfused through the stem. These solutions were introduced through F with stopcock B closed. With pressures from 5 to 10 pounds per square inch the vessel sap could be replaced in from 8 to 28 hours depending upon the individual stem and its previous history. Burette G was used to supply the stem with distilled water or a sucrose solution during the cooling and freezing process.

Results

The data presented here are the results of experiments to explore the variations of flow responses from maple stems subjected to a variety of treatments under controlled temperatures. The flows were induced by rais-

ing the temperatures usually to 7° C, until flow ceased. In no experiment was a constant volume maintained at the end of a flow, but rather absorption followed immediately.

The experiments were usually run with six stems in replicate. These stems were collected from different individual plants and were selected for uniformity of shape and weight. The only source of material was naturally grown plants. Although grown in a fairly uniform stand, such plants probably show genetical differences as well as the effects of variation in the composition and moisture content of the soil.

At the outset it is of interest to compare the sap flow of sugar maples with that of another species of maple and other genera commonly associ-

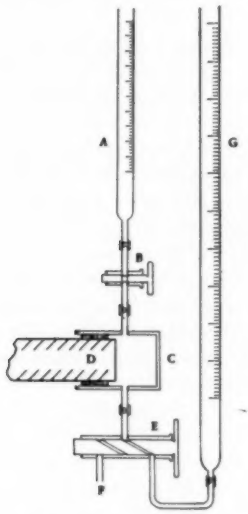

FIG. 1. Apparatus used to measure sap flow. See the discussion in the text.

ated with sugar maple in nature. Five stems, two from species of Acer and three from other genera, were given the same treatment—that is, frozen and then warmed to 7° C. Under these conditions both species of Acer gave a flow while the other genera absorbed water. These results are illustrated in table I.

In reviewing the results of a number of experiments in which the experimental conditions were essentially the same—that is, the stems, after being assembled in the apparatus and absorbing water on cooling, were frozen and then warmed to 7° or 8° C—a great variation in the flow pattern became apparent. It may be seen from table II that there is a variation in the duration of the flow from different stems from 1.25 hours to 53.25 hours. In general, freshly cut stems flow at a rapid rate although for a relatively short

TABLE I

FLOW RESPONSE (IN MILLILITERS) OF STEMS OF FOUR GENERA AND FIVE SPECIES AFTER FREEZING AND THAWING.

Experiment number	*Acer pennsylvanicum*	*Acer saccharum*	*Betula lutea*	*Prunus serotina*	*Fagus grandifolia*
	ml.	*ml.*	*ml.*	*ml.*	*ml.*
1	+2.6	+2.1	- 5.6	-0.5	-1.5
2	+1.5	+2.9	-10.9	-1.8	-8.8
3	+0.2	+1.8	- 5.6	-5.2	-5.5
Average	+1.4	+2.3	- 7.4	-2.5	-5.3

time as compared with stems which have been held frozen in storage for several weeks. In both types of material there is no apparent correlation between the duration of the flow and the total amount of sap produced. There may be both large and small amounts of sap produced during short flow periods and long flow periods.

Other variations observed were the differing times and temperatures at which flows began. Very commonly as the stems thawed there was an initial decrease in volume in the measuring system, perhaps the result of the change in state from solid to liquid in the vessels. If a flow occurred, this initial decrease was followed by an increase in volume in the measuring system. Table III gives the data from a typical experiment in which the experimental box was warmed to 7° C and held at that temperature. The data represent the cumulative changes in volume. During the three and a half hours required to warm the system, the stems warmed at a slower rate than the experimental box. Stem 3 began a flow after being warmed 1.0° C while stem 4 did not begin to flow until it had been warmed 5.5° C. Stems 1 and 2 were intermediate and, as often happened, stems 5 and 6 continued to absorb for the entire experimental period. Similar data are presented in table

TABLE II

VARIATIONS IN THE AMOUNT AND DURATION OF FLOW FROM DIFFERENT STEMS.

Stem weight	Month	Condition of stem	Temperature during flow	Duration of flow	Flow
gm.			*°C*	*hrs.*	*ml.*
459	December	Freshly cut	8	1.25	1.1
735	March	" "	8	1.50	6.8
915	March	" "	8	1.50	14.9
970	March	" "	8	2.00	17.3
627	November	" "	7	3.50	2.3
734	July	Stored stem	7	6.00	6.8
652	June	" "	7	14.75	7.2
583	July	" "	7	19.00	4.3
699	June	" "	7	26.50	21.6
621	June	" "	7	26.50	1.8
540	June	" "	7	28.00	1.0
477	June	" "	7	53.25	15.7

TABLE III

UNDER CONDITIONS OF RISING TEMPERATURE (TO 7°C) THERE ARE VARIATIONS BETWEEN STEMS IN THE TEMPERATURES AT WHICH FLOWS BEGIN (MAY).

Time	Box temp.	Stem temp.	Volume change in milliliters					
			Stem no. 1	2	3	4	5	6
min.	°C	°C						
0	+0.5	+0.5	0.0	0.0	0.0	0.0	0.0	0.0
15	+5.0	+1.5	−0.3	−0.1	+0.1	−0.1	−0.1	−0.5
30	+6.0	+2.0	−0.4	−0.2	+0.1	−0.1	−0.3	−0.7
45	+7.0	+4.0	+0.2	−0.3	+0.1	−0.2	−0.5	−1.1
60	+7.5	+5.0	+0.8	−0.3	+0.2	−0.2	−0.6	−1.3
75	+7.5	+5.5	+1.7	−0.1	+0.3	−0.2	−0.6	−1.5
90	+6.5	+5.5	+2.2	+0.1	+0.6	−0.1	−0.6	−1.7
105	+6.5	+6.0	+2.8	+0.2	+0.9	0.0	−0.6	−1.8
120	+7.0	+6.0	+3.3	+0.4	+1.1	0.0	−0.7	−2.0
135	+7.0	+6.0	+3.8	+0.5	+1.3	+0.2	−0.7	−2.1
150	+7.0	+6.5	+4.0	+0.7	+1.5	+0.2	−0.7	−2.3
180	+7.0	+6.5	+4.3	+0.9	+1.7	+0.3	−0.8	−2.4
195	+7.0	+6.5	+4.6	+1.1	+1.9	+0.4	−0.8	−2.5
210	+7.0	+7.0	+4.7	+1.3	+2.0	+0.5	−0.9	−2.6
225	+7.0	+7.0	+4.9	+1.4	+2.1	+0.5	−0.9	−2.7

IV, except that the final temperature was 29.5° C. Here stem 8 began flowing after a temperature rise of 5.5° C and continued to flow for 15 hours. Stem 10 also started to flow after a rise of 5.5° C but after two hours began to absorb. Stem 9 did not flow until after the temperature was elevated to 29.5° C. The other stems began to flow at temperatures between these

TABLE IV

UNDER CONDITIONS OF RISING TEMPERATURES (TO 29.5° C) THERE ARE VARIATIONS BETWEEN STEMS IN THE TEMPERATURE AT WHICH FLOW BEGINS (JULY).

Time	Box temp.	Stem temp.	Volume change in milliliters					
			Stem no. 7	8	9	10	11	12
hrs.	°C	°C						
.0	−0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.5	+12.0	+5.5	−0.5	+0.6	−0.5	+0.1	−0.5	−0.8
1.0	+14.5	+13.0	−0.5	+1.7	−0.5	+0.6	+0.4	−0.6
1.5	+15.5	+15.5	−0.4	+2.6	−0.7	+0.7	+1.2	−0.6
2.0	+18.0	+17.0	−0.3	+3.4	−0.9	+0.6	+1.3	−0.7
2.5	+20.0	+18.0	−0.2	+4.3	−0.9	+0.5	+1.3	−1.0
3.0	+21.0	+21.0	+0.1	+5.3	−1.1	+0.3	+1.2	−1.1
3.5	+23.0	+23.0	+0.4	+6.3	−1.2	+0.2	+1.0	−1.0
4.5	+25.5	+25.5	+3.0	+9.4	−1.4	+0.2	+0.7	+0.9
8.0	+28.0	+28.0	+4.1	+11.1	−0.9	+0.3	+0.8	+2.9
9.0	+29.5	+29.5	+5.3	+11.8	0.0	−0.3	+1.0	+3.9
11.25	+29.5	+29.5	+5.4	+12.8	+1.6	−0.2	+0.9	+5.3
12.5	+29.5	+29.5	+6.2	+13.3	+2.1	−0.3	+0.7	+5.5
14.0	+29.5	+29.5	+7.1	+13.7	+2.6	−0.5	+0.4	+5.6
15.0	+29.5	+29.5	+7.5	+13.8	+2.9	−0.7	+0.3	+5.5

TABLE V

VARIATIONS IN FLOW BETWEEN TWO SECTIONS OF THE SAME STEM (DECEMBER).

Basal section			Upper section		
Stem weight	Volume change at 7° C	Flow per 1000 g. stem	Stem weight	Volume change at 7° C	Flow per 1000 g. stem
gm.	*ml.*	*ml.*	*gm.*	*ml.*	*ml.*
957A	+ 3.5	+ 3.7	609A	+ 1.5	+ 2.5
1007B	+ 1.3	+ 1.3	595B	+ 7.3	+ 12.3
987C	+ 7.0	+ 7.1	628C	+ 2.1	+ 3.3
736D	- 1.0	- 1.4	422D	+ 0.1	+ 0.2
853E	+ 1.7	+ 2.0	525E	- 1.7	- 3.2
706F	+ 4.5	+ 6.4	1054F	- 1.3	- 1.2
*874.3**	*+ 2.6*	*+ 3.3*	*638.8*	*+ 1.3*	*+ 2.3*

* Figures in italic represent averages.

extremes. From these data it is clear that all stems do not show the same response to changes in temperature.

Not only do the flow characteristics of individual plants differ when compared under uniform conditions, but sections of the same plant differ from each other. Six stems were selected and each stem cut into two sections, a basal section and an upper section. From table V, stem weights 957A and 609A are the weights of the basal and upper sections of the same stem. When the flows from the two sections, as for example stem C, were compared on a weight basis the flow for the basal section was 7.1 ml./1000 g. while for the upper section it was 3.3 ml./1000 g. In contrast, the upper section of stem B gave a higher yield than the basal section. A more striking difference than the quantitative one is the difference in direction of the response as in stems E and F. Here the basal sections gave a flow while the upper sections absorbed. It is clear that more must be known of the flow mechanism before reproducible results on a quantitative basis can be obtained.

TABLE VI

WITH STANDARD TREATMENT STEMS CUT DURING ACTIVE GROWTH (AUGUST) DO NOT FLOW.

Stem weight	Water absorbed on freezing	Volume change at 7° C
gms.	*ml.*	*ml.*
522	100.0	- 14.0
567	51.2	- 6.0
515	65.8	- 16.3
430	91.6	- 9.8
652	70.6	- 11.1
530	103.2	- 19.7
*536**	*80.4*	*- 12.8*

* Figures in italic represent averages.

TABLE VII

A SECOND FLOW PRODUCED BY RAISING THE TEMPERATURE AFTER A FLOW AT A LOWER TEMPERATURE.

Log weight	Volume change at the lower temperature	Volume change at the higher temperature
gm.	*ml.*	*ml.*
	Experiment 9 December	
Duration	10 hrs.	3.5 hrs.
Temperature	7° C	17° C
642	− 5.4	− 4.5
667	+ 12.3	+ 1.7
632	+ 6.5	− 1.2
565	+ 7.7	− 1.0
681	+ 12.3	+ 1.3
558	+ 4.2	+ 1.3
*624.1**	*+ 6.3*	*− 0.4*
	Experiment 10 December	
Duration	6.5 hrs.	3 hrs.
Temperature	7° C	17° C
636	+ 0.9	+ 0.1
748	+ 4.9	+ 2.9
636	+ 6.1	+ 1.9
677	+ 4.2	+ 0.4
791	+ 0.6	− 1.4
515	+ 1.4	+ 1.4
667.1	*+ 3.0*	*+ 0.9*
	Experiment 11 December	
Duration	6 hrs.	3 hrs.
Temperature	7° C	17° C
697	+ 12.6	+ 2.2
617	+ 3.9	+ 1.2
608	+ 8.0	+ 2.0
577	+ 4.6	− 3.2
700	+ 2.9	− 1.3
459	+ 1.1	+ 0.4
609.6	*+ 5.5*	*+ 0.2*
	Experiment 12 January	
Duration	3.5 hrs.	3 hrs.
Temperature	7° C	25.5° C
625	+ 1.9	+ 2.2
872	− 2.0	− 1.6
819	+ 0.1	+ 0.3
627	+ 2.9	+ 2.3
518	+ 2.7	+ 1.4
872	+ 1.0	+ 2.5
722.1	*+ 1.1*	*+ 1.2*

* Figures in italic represent averages.

As a consequence, in the following experiments the interpretations of the results will be based on qualitative differences—that is, whether flow or absorption occurs—rather than on the quantitative measure of the flow observed.

The question arose whether under experimental conditions a flow might be obtained from stems collected in midsummer. Six stems were collected in August and on freezing and thawing they absorbed (table VI). Two possible explanations of these results have been suggested: first, that the cells in active growth were injured by freezing; second, that the starch con-

TABLE VIII

THE INACTIVATION OF THE FLOW MECHANISM BY STEAM.

Log weight	Volume change in normal flow	Volume change after exposure to steam at 1 p.s.i. for 2 hours
gms.	*ml.*	*ml.*
	Experiment 13 December	
Duration	7.5 hrs.	5.5 hrs.
514	− 4.9	− 8.4
611	+ 10.0	− 0.6
593	+ 6.8	− 1.6
559	+ 2.1	+ 0.3
571	+ 11.6	− 0.9
459	+ 6.0	− 0.4
*551.1**	*+ 5.3*	*− 1.9*
	Experiment 14 December	
Duration	5.75 hrs.	10 hrs.
697	+ 13.5	− 1.6
617	+ 4.9	− 7.3
608	+ 8.0	− 5.0
577	+ 4.6	− 8.0
700	+ 3.0	− 3.2
459	+ 1.1	− 6.5
609.6	*+ 5.9*	*− 5.3*

*Figures in italic represent averages.

tent is high and the sucrose content low in the wood at this time of year. Either or both of these factors might account for the lack of flow observed. Other observations indicate that maple stems will not flow well until four to six weeks after leaf fall.

In a consideration of the flow mechanism two points of view have been expressed in the literature: first, that the flow is a thermal response of a physical system or systems; second, that it is a temperature response of a biological mechanism or mechanisms.

In nature the flow of maple sap occurs during the time of year when the diurnal temperature range is frequently above and below 0° C. Does the

flow mechanism require freezing temperatures? Previous workers have suggested that it does. The question was reinvestigated in four experiments. The stems were set up in the usual manner in the experimental box, frozen, and allowed to flow at 7° C after freezing. At the end of the flow, when all of the stems had begun to reabsorb as with tree 10, table IV, the temperature was raised to 17° C in three of the experiments and to 25.5° C in the fourth. From table VII it is evident that a second flow occurred in many stems. Thus it was clear that freezing is not a necessary requirement for the flow mechanism. Field observations during the spring of 1949, based on the thermocouple temperatures of the phloem and outer xylem of a number

TABLE IX

THE FLOW RESPONSE FROM MAPLE STEMS PERFUSED WITH DISTILLED WATER AND WITH SAP FROM OTHER TREES.

Stem weight	Flow before perfusion	Flow/kg. log weight	Ml. water perfused	Flow	Sap perfused	Flow
gms.	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*
			Experiment 15 March			
Duration	3.5 hrs.		26.5 hrs.	3.3 hrs.	22 hrs.	2 hrs.
970	+17.3	+17.8	532	-14.3	202	+4.4
771	+ 9.6	+12.5	188	- 7.7	44	+1.5
735	+ 6.8	+ 9.3	292	-12.1	88	+4.1
860	+ 8.4	+ 9.8	445	-18.3	144	+1.0
895	+13.9	+15.5	373	-14.9	180	+3.7
915	+14.9	+16.3	409	-13.9	154	+2.2
*858**	*+11.8*	*+13.5*	*373*	*-13.5*	*135*	*+2.8*
			Experiment 16 November			
Duration	6 hrs.		17 hrs.	15 hrs.	24 hrs.	3 hrs.
648	- 1.3	- 2.0	184	-15.4	40	-0.3
627	+ 2.2	+ 3.5	200	- 4.4	40	+0.4
677	+ 7.2	+10.6	190	-14.0	122	+0.3
585	- 4.5	+ 7.7	220	- 9.1	76	-0.4
584	- 5.0	- 8.6	650	-16.9	150	+1.8
587	+ 3.6	+ 6.1	108	- 2.7	60	+2.9
618	*+ 0.4*	*+ 2.9*	*259*	*-10.4*	*81*	*+0.8*
			Experiment 17 November			
Duration	6 hrs.		24 hrs.	21.5 hrs.	21 hrs.	5 hrs.
576	+ 3.3	+ 5.7	80	- 6.1	50	-0.2
705	+11.3	+16.0	173	- 9.3	118	+2.9
774	+ 9.8	+12.7	318	-17.9	146	+0.9
644	+ 9.1	+14.1	132	- 8.0	90	+1.6
740	+ 0.5	+ 0.7	56	- 9.3	68	+0.3
884	+ 8.6	+ 9.7	213	-16.2	172	+7.2
721	*+ 7.1*	*+ 9.8*	*162*	*-11.1*	*107*	*+2.1*

* Figures in italic represent averages.

of trees, clearly demonstrated that good flows occurred when the lowest recorded stem temperatures of the diurnal cycle were appreciably above 0° C.

The second point of view was investigated by treating the stems with steam after a normal flow. The stems were removed from the experimental box and treated with live steam at one pound per square inch for two hours in a steam box. This was considered sufficient to inactivate the living cells. The stems were then allowed to absorb, were frozen and warmed to 7° C as before. From table VIII it can be seen that after the steam treatment no

TABLE X

FLOW CHARACTERISTICS OF STEMS PERFUSED WITH A 5% SUCROSE SOLUTION.

Log weight	Sucrose perfused	% solids in perfusate	Volume change at 7°C
gms.	*ml.*	%	*ml.*
	Experiment 18 November		
Duration	22 hrs.		4 hrs.
632	315	4.6	+24.3
638	260	4.3	+23.7
670	380	4.5	+ 5.6
710	450	4.6	+30.5
688	305	4.3	+28.2
800	450	4.5	+ 7.4
*689.6**	*360*	*4.5*	*+20.0*
	Experiment 19 December		at 24°C
Duration	16.25 hrs.		3 hrs.
673	140	5.0	+10.8
731	225	5.0	+10.8
860	295	5.0	+ 0.3
844	600	5.0	+12.3
733	460	5.0	+ 0.8
708	260	5.0	+ 1.1
758.1	*330*	*5.0*	*+ 6.0*

*Figures in italic represent averages.

flow occurred except for one doubtful case; rather, the stems absorbed water. Thus, it is evident that the activity of living cells is necessary to produce a flow.

In an attempt to study the internal conditions necessary for sap flow, the stems were perfused with distilled water under pressure, after a trial run with no treatment. The perfusion was continued until there was a negative test for solids in the perfusate with a refractometer. For example, a stem weighing 771 grams gave a negative test after 188 ml. of water had been perfused (table IX, experiment 15). When stems were perfused under uniform pressure there was great variation in the rate of perfusion of different

stems. Thus, during a perfusion period of 26.5 hours (table IX, experiment 15), 188 ml. of water passed through one stem while 532 ml. was perfused through another. After perfusion the stems were frozen and warmed to 7° C. In contrast to the normal flow, all stems absorbed. Thus, replacing the vessel sap with distilled water reversed the direction of the flow mechanism. This experiment has been repeated several times with the same results. It should be pointed out that the period of perfusion is long, from 17 to 26 hours, and some materials may diffuse from adjacent cells into the vessels during the perfusion. However, the uniformly negative results indicate that

TABLE XI

FLOW CHARACTERISTICS OF STEMS PERFUSED WITH A MANNITOL SOLUTION.

Stem weight	Volume change without treatment 7 °C	Ml. mannitol perfused	% solids in perfusate	Volume change at 7° C
gms.		*ml.*	*%*	*ml.*
		Experiment 20 January 2.5% Mannitol		
Duration		24 hrs.		6.5 hrs.
859		425	2.5	- 22.0
892		165	2.2	- 20.5
747		230	2.4	- 13.2
877		490	2.5	- 20.2
828		290	2.4	- 16.6
838		360	2.4	- 25.7
*840.1**		*326.6*	*2.4*	*- 19.7*
		Experiment 21 January 2% Mannitol		
Duration		21 hrs.		2.5 hrs.
660	+ 3.4	62.5		- 16.2
665	+ 1.7	500		- 13.2
865	+ 2.3	590		- 17.0
920	+ 0.5	850		- 11.2
1070	+ 5.5	990		- 23.7
780	+ 3.9	940		- 12.3
826.6	*+ 2.9*	*749.1*		*- 15.6*

* Figures in italic represent averages.

they are not present in sufficient quantity to influence the response to elevated temperatures. The same stems were next perfused with freshly collected sap from other trees. After freezing and warming a flow occurred. The flow observed was, however, somewhat less than the initial one. It is interesting to note that one stem in experiment 16 which did not flow with its own sap did flow with the sap from another tree.

From these experiments it became clear that perfusing with a synthetic sap would be of interest and, since sucrose is the largest single component of natural sap, a sucrose solution was used. Previous experiments have demon-

strated that 200 ml. is a sufficient quantity of perfusate to replace the vessel sap (table IX). Twelve stems in experiments 18 and 19, table X, were perfused with a 5% sucrose solution. The stems were then frozen and in experiment 18 warmed to 7° C. In all cases a flow occurred and in some stems in a considerable amount. The conditions in experiment 19 were the same as in experiment 18 except that the stems were warmed to 24° C. Replacing the sap with a sucrose solution, therefore, does not inactivate the flow mechanism since an apparently normal flow occurs after such treatment.

The preceding experiment gives rise to the question of whether the presence of sucrose in the vessel sap is essential for a flow. To investigate this question stems were perfused in some cases with a 2.5% mannitol solution and in others with a 2% mannitol solution. These concentrations are essentially isosmotic with the sucrose concentration commonly found in the stems. In table XI, experiment 20, good perfusion was obtained in 24 hours and in experiment 21, in 21 hours. In both experiments, after perfusion and the subsequent freezing and thawing, there was a very considerable absorption rather than flow. This absorption was nearly twice that with distilled water in experiments 15, 16 and 17, table IX.

Discussion

The data clearly indicate that flows from comparable stems under uniform temperature conditions are not quantitatively reproducible. Repeated flows from the same stem under the same temperature conditions result in different amounts of flow. Flows from different stems or from sections of the same stem under the same temperature conditions are widely different quantitatively, when compared on a fresh weight basis, and may even differ qualitatively. In other words, some flow while others absorb. For the present the significance of the experiments is related to the direction of the flow mechanism—whether a flow or absorption occurs. A more complete understanding of the flow mechanism is necessary to interpret the quantitative differences.

Theoretical considerations of possible explanations of the flow mechanism fall into two groups. The first concerns the effect of the temperature change on a physical system—namely, thermal expansion of gases, of the vessel solutions, or of the cell walls. The other point of view is directed at the effect of temperature change on a biological system. A thermal gradient along a ray cell or between ray cells might result in differences in the concentration of osmotically active substances or differences in membrane permeability or both. It has also been suggested that temperature differences would change the oxygen concentrations in the vessel sap and in the cells thus influencing respiration which in turn would influence flow.

Some of the results in tables III and IV are of interest in considering thermal expansion of a physical component as a direct cause of pressure and flow. In table III, stem 4 did not begin to flow until it had warmed to within 1° C of the final elevated temperature while stem 3 began to flow

after a rise of 1° C. Stem 9, table IV, did not begin to flow until the stem was warmed to the holding temperature. If the cause of flow were the expansion of a physical system alone, more uniformity in the response would be expected. The data in table VII are of interest in that a flow may be produced by a rise in temperature from a point 7° C above 0° C. Thus freezing is not a necessary antecedent for a flow. Thermal expansion of the system undoubtedly plays a part but does not appear to explain the entire mechanism.

The suggestion of a thermal gradient along living ray cells as a causative factor in the flow mechanism is open to doubt when the temperatures at which flow occurred in stems 7, 9, and 12, table IV, are considered. The stem temperatures were recorded with thermocouple points inserted into the center of the stem. It may be seen that the internal temperatures were the same as that surrounding the stems—that is, stem temperatures were uniform throughout at the time flow began.

Treating the stems with steam and presumably inactivating the living cells reversed the direction of movement in the vessels, thereby suggesting that the flow mechanism is a biological phenomenon.

The composition of the vessel sap profoundly influences the flow mechanism: tables IX, X, and XI. The direction of movement was reversed when distilled water replaced the vessel sap; sap from other trees or a sucrose solution perfused into the vessels resulted in an outward flow. The amount of sap produced was not, however, directly correlated with the sucrose concentration in the vessel sap (experiment 15, table IX). Extensive field observations by F. H. Taylor to be published elsewhere are in agreement.

Perfusion with a mannitol solution isosmotic with the sucrose concentrations usually found gave results in flow behavior unlike that with sucrose but similar to that with distilled water. This result indicates that either there is rapid absorption of the mannitol solution from the vessel sap, or flow is not the result of a simple osmotic mechanism. Experiments are in progress in which stems are being perfused with a number of different sugars to determine whether sucrose is a specific requirement.

Summary

1. A method has been developed to measure the flow of sap from small maple stems under controlled temperatures.

2. There is great variation in the quantity of sap produced under uniform temperature conditions from similar stems, or even from sections of the same stem.

3. A second flow of sap may be obtained by a second temperature elevation after the first flow following freezing. This fact indicates that a freeze before each flow is not necessary.

4. Flows are produced by stems perfused with sap from other trees or with a sucrose solution.

5. Absorption occurs when the stems are perfused with distilled water or a mannitol solution isosmotic with the sucrose content of normal sap.

6. Thermal changes in a physical system alone are not sufficient to account for the flows observed.

7. Living tissues are necessary for the flow mechanism to function.

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STOMATAL MOVEMENT AND PHOTOSYNTHESIS IN PELARGONIUM. II. EFFECTS OF WATER DEFICIT AND OF CHLOROFORM: PHOTOSYNTHESIS IN GUARD CELLS[1]

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(WITH FIVE FIGURES)

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This paper describes the second half of a series of tests of the photosynthetic theory of stomatal movement. A corollary of this theory is that any agent that inhibits photosynthesis should prevent opening or cause closure in light—unless some other action prevents this. It has been reported that this relationship does not hold either in wilting or under the action of chloroform (WILSON, **14**). The effects of these factors are investigated in the present paper. As before (**11**), stomatal aperture was measured by means of a modified porometer method, photosynthesis by infrared absorption, and, except in a few experiments, the leaf material all belonged to the same clone of *Pelargonium zonale* var. Barney.

Water

The water balance of the leaf may act on stomata either directly through its effects on the turgidity of the guard cells and on epidermal tissue tensions (passive movement), or indirectly through its effect on physiological activities within the guard cells (active movement). In testing the photosynthetic theory we had to study both types because they interact.

WILTING OF SEVERED LEAVES AT DIFFERENT LIGHT INTENSITIES AND WATER VAPOR PRESSURE DEFICITS

The purpose was to compare the courses of stomatal closure and decline of photosynthesis after the leaf petiole had been cut, while varying the conditions so as to produce different initial rates of photosynthesis and transpiration. The various conditions and data regarding the leaves themselves are given in table I. Each leaf was allowed to come to equilibrium with its respective conditions and then the petiole was cut. The consequent courses of photosynthesis and of stomatal mean diameter are shown in figure 1. Apart from initial "passive opening" the stomatal curve has a broad resemblance to that of photosynthesis for each particular leaf. It is clear, however, that this agreement cannot simply be the result of stomatal control of photosynthesis. The latter began to decline while the stomata were still opening, it reached a low level by the time the stomata returned to their original aperture, and became zero before closure was complete.

[1] Contribution from the Faculty of Agriculture, McGill University, Macdonald College, Quebec, Canada. Journal Series no. 284.

TABLE I

EXPERIMENTAL CONDITIONS OF LEAVES SUBJECT TO DIFFERENT VAPOR PRESSURE DEFICITS (v.p.d.) AND LIGHT INTENSITIES (L.I.) WHEN THEIR PETIOLES WERE CUT (See fig. 1).

Leaf	L.I. fc	v.p.d. mm. Hg.	Air flow l/h	Leaf area μ^2	Stomata per cm^2	Principal feature of the experiment
O	500	14.8	40	67.5	19,900	Low v.p.d. intermediate L.I.
P	3000	28.5	44	86.5	22,700	High v.p.d. high L.I.
Q	500	27.2	45	74.2	16,400	High v.p.d. intermediate L.I.
R	360	26.0	55	85.0	17,900	High v.p.d. low L.I.

These curves will be discussed later in relation to others. Meantime it may be pointed out that the relatively slower progress of the wilt-induced changes in leaf O can be ascribed to the lower v.p.d., and that in the others (kept at nearly equal v.p.d.) the speed with which the photosynthesis curves drop before the stomatal curves have returned to their initial level is R > Q > P, which is the reverse of the order of light intensity. This fact shows that the drop in photosynthesis was not due to a reduction in permeability of the cell walls to CO_2 as a result of their loss of water. In sunlight (P) the rate of photosynthesis before cutting was certainly limited mainly by the supply

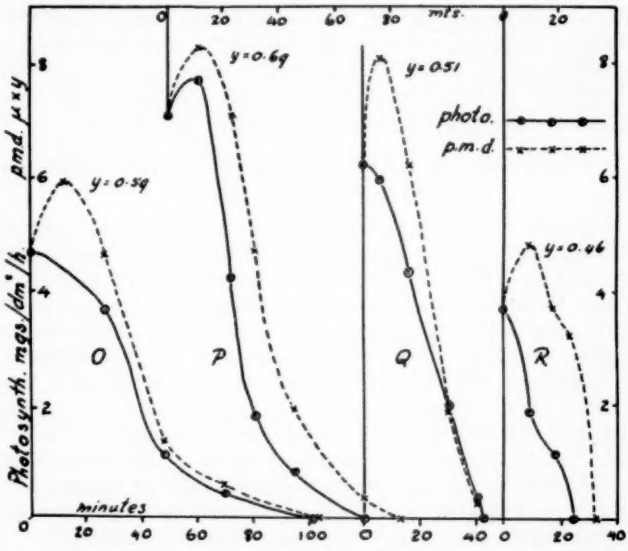

FIG. 1. Time courses of photosynthesis rate and pore mean diameter following cutting of the leaf petiole under different conditions (table I). y is the factor by which p.m.d. is multiplied to make its starting point coincide numerically with that of photosynthesis.

of CO_2, so that a further reduction in its supply would have produced a more immediate decline in the rate than where CO_2 was not limiting. On the contrary the first change in photosynthesis in this case was a slight increase, due no doubt, to the passive stomatal opening. We must suppose, therefore, that reduction in water content of the cells inhibits photosynthesis in some other way not yet understood.

Comparison of the effects of CO_2-free air and normal air on stomatal closure during wilting

Two leaves, S and T, were brought to equilibrium with light of 500 fc and then cut off from their water supply as before, accompanied, however, by simultaneous replacement of normal air by CO_2-free air and in S, by extinction of the light. Comparing the wilting curves of S and T (fig. 2)

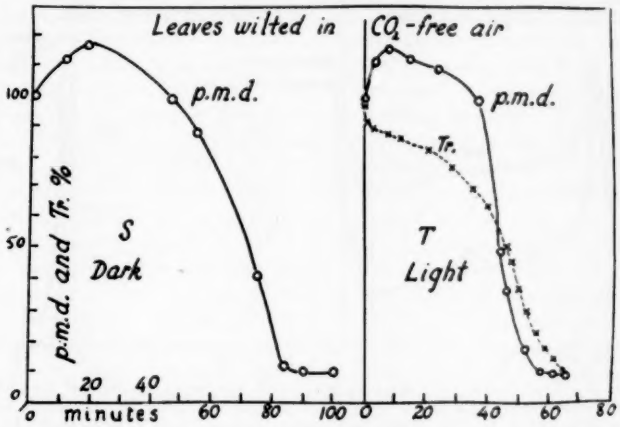

Fig. 2. Time courses of stomatal pore mean diameter (p.m.d.) following cutting of the leaf petiole in CO_2-free air, leaf S in darkness, leaf T in light. The course of transpiration (Tr) of leaf T is shown also.

with one another, the earlier completion of closure in T is ascribable to the higher v.p.d., 21.2 mm. Hg compared with 16 in S. The only difference that may be due to light is the flatter top of curve T.

For comparison with normal air we may take O and Q in figure 1, since T was wilted under the same light as these and at a v.p.d. that was about midway between theirs. It may be seen that in CO_2-free air the first part of closure proceeded more slowly. By the time (about 38 min.) that the p.m.d. had just returned to its initial size (100%) in T, and was still greater than this in S, it had fallen to about 50% in O and 10% in Q. During the later stages of wilting in CO_2-free air closure was accelerated, but at the end it stopped rather abruptly while still short of completion. The persisting apertures in each of S and T had an average mean diameter of about 1.2 μ.

To compare behaviors in normal and CO_2-free air of the same leaf as affected by water balance, the next experiment was made on a leaf attached to the plant. Under light of 500 fc and at high temperature, 35.5° C, a sudden switch was made from fairly moist to completely dry air, and this was repeated later with CO_2-free air. The results are shown in figure 3.

With normal air the sudden increase of v.p.d. resulted in the usual passive opening followed quickly by a rapid closing movement, which soon slowed down, presumably as closure allowed the water balance of the leaf to be restored. After about four hours, during which period the leaf still seemed to be losing water slowly, since the closing movement continued, a return to moister air coupled with removal of CO_2 induced rapid opening. As soon as the original stomatal aperture (though probably not the original

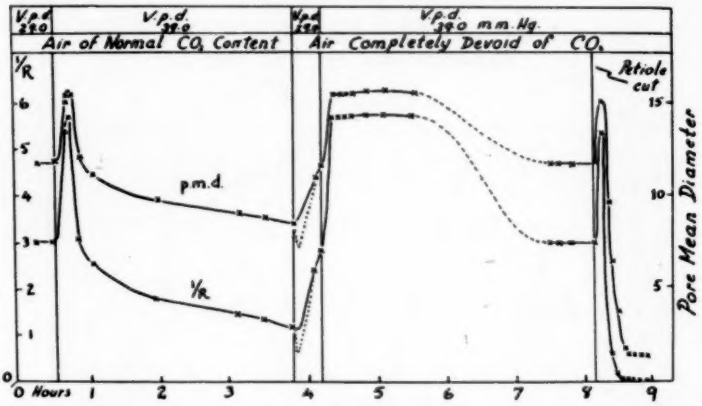

FIG. 3. Time courses of 1/R (pore area) and pore mean diameter of an attached leaf as affected by changes in the vapor pressure deficit of the air stream, first in normal air and then in CO_2-free air. Other conditions constant. L.I., 1000 fc; leaf temperature, 35.5° C; air flow, 70 l./h.

water balance because of the CO_2 factor in opening) was reached the air was again dried. Passive opening occurred as before but this time the peak was maintained for at least 75 minutes, despite the fact that the combination of wide opening and dry air must have considerably reduced the water balance. Eventually, during an interruption of the measurements, partial closure occurred and a new equilibrium seemed to be established.

When, finally, the leaf petiole was cut to intensify wilting, stomatal closure proceeded rapidly to its apparent limit, which as in S and T was a condition of slight stomatal slits. It might be added that examination of the guard cells revealed no increase in starch content over that in their originally open condition.

Let us try to interpret these wilting curves from the standpoint of mechanism. The initial passive opening, if due to a temporary upset in the water balance between the guard cells and adjoining cells, would soon be reversed

in absence as well as presence of CO_2. The fact that closure may be long delayed in CO_2-free air (fig. 3) indicates not only that the opening is probably due to a general loss of turgor, but also that the closure in normal air is of the active type, for the most part at least. The further finding that it follows closely upon a parallel decline in photosynthesis (fig. 1) is in harmony with the view that accumulation of CO_2—largely prevented in absence of external CO_2—is an intermediate factor between wilting and stomatal closure.

As regards closure in CO_2-free air, it was found earlier (11) that the absence of a condition necessary for photosynthesis, namely, light or chlorenchyma, is associated with less wide opening than when all the necessary conditions are present, though it is only respiratory CO_2 that is thereby reduced. It follows, if the theory applies, that inhibition of photosynthetic activity in CO_2-free air as the result of wilting should reduce stomatal opening to a similar extent. There is evidence of this in the partial closure which took place in the attached leaf during exposure to dry CO_2-free air (fig. 3). This reduction may have begun earlier than is apparent, being obscured by continuation of passive opening. But in any case it would start later than in normal air because, with CO_2 a strongly limiting factor in photosynthesis at the start, wilting had to proceed much further before water could become limiting. Closure ended when stomatal aperture was reduced to about the level it would have reached if photosynthesis had been stopped by darkness instead of wilting. In the rapid and progressive wilting of the cut leaf T (fig. 2) no such equilibrium could be established, but the phase of inhibition of photosynthesis was probably synchronous with the "shoulder" of the stomatal curve. In S, where the leaf was darkened at the start, the shoulder of the curve is more sloping, in spite of the lower v.p.d. and rate of wilting.

A third phase of movement seems to develop at an advanced stage of wilting. We refer: (a) to the extremely rapid closure that follows cutting the petiole—after photosynthesis is inferred to have stopped (fig. 3); (b) to the rather sudden acceleration of closure in the middle of its course in leaf T, less marked in S (fig. 2); and (c) to the speedy completion of closure in some of the leaves wilted at high v.p.d. in normal air (fig. 1). All these are in contrast with the gradual tapering off of closure shown by leaf O kept at a lower v.p.d. It seems likely that in this last case the active mechanism discussed above was able to complete the closure before water loss reached the stage that produces the accelerated type, and that the latter, since its rate is apparently limited only by the rate of wilting, may have a passive mechanism. This hypothesis suggested the study of wilt closure through another type of experiment.

Starch content and posture of guard cells under various conditions of opening and closure

Microscopic measurements were made of the starch content of the guard cells and of the dimensions of the stomatal apparatus, as well as of the pore, in wilt-closed as compared with light-open and dark-closed stomata. Three

variations in the mode of wilting and five different leaves were used. Experimental results are given in table II. The measurements, which were made on fixed epidermal strips, and some calculations from them are also given in tables II and III.

Starch content (based on iodine tests made after measurement of dimensions) was estimated according to four grades from I, traces of starch, to IV, a heavy deposit. There was no increase in starch content over the light open grade in the case of the leaf wilted in CO_2-free air or in that of epidermal

TABLE II

STARCH CONTENT AND PORE DIMENSIONS OF GUARD CELLS IN (1) LIGHT-OPEN, (2) WILT-CLOSED AND (3) DARK-CLOSED STOMATA. LIGHT INTENSITY 1000 fc. TEMPERATURE 28–32° C. AIR FLOW 70 l/h. NORMAL v.p. OF AIR REPRESENTS A v.p.d. OF 20–26 mm. Hg. WILT CUT: PETIOLE CUT AND LEAF WILTED UNDER LIGHT IN COMPLETELY DRY AIR (FOR 30–38 MINUTES) UNTIL STOMATAL CLOSURE CEASED. WILT STRIP: EPIDERMAL STRIPS TAKEN FROM A FRESH LEAF AND EXPOSED TO RELATIVELY DRY LABORATORY AIR (FOR 2 TO 5 MINUTES) UNTIL CLOSURE CEASED.

Leaf	Experimental conditions				Stomatal pore μ		
	General	Cutting	CO_2	V.P.	Starch grade	Length	Width
V	1. Light	Uncut	Normal	Normal	I	12.20	5.70
	2. Wilt	Cut	Normal	0	II	....*	....
	3. Dark	Uncut	Normal	Normal	IV	0	0
W	1. Light	Uncut	0	Normal	I	13.52	8.23
	2. Wilt	Strip	0	Low	I	....	....
	3. Dark	Uncut	Normal	Normal	IV	0	0
X	1. Light	Uncut	0	Normal	I	12.5	6.8
	2. Wilt	Cut	0	0	I	....	....
	3. Dark	Uncut	Normal	Normal	IV	0	0
Y	1. Light	Uncut	Normal	Normal	I	12.1	4.0
	2. Wilt	Strip	Normal	Low	I	....	....
Z	1. Light	Uncut	Normal	Normal	I	10.6	9.0
	2. Wilt	Strip	Normal	Low	I	....	....

* The pore in wilt-closed stomata was a slit less than 1 μ wide.

strips wilted within a few minutes in normal air, but in the case of the leaf wilted to the point of stomatal closure during 38 minutes in normal air, the starch content rose distinctly to grade II. The inference is that in this last case the active mechanism, involving a sugar to starch transformation, contributed in some measure to the closing movement, but that during the very rapid wilting of thin strips there was insufficient time for it to do so, and in CO_2-free air it was unable to operate irrespective of time. It seems clear therefore that at a severe degree of wilting some kind of passive mechanism tending to produce stomatal closure comes into play, whether the air contains CO_2 or not.

A comparison of the dimensions of the stomatal apparatus (table III) showed that in all types of wilt closure the shape of the stoma was in certain respects intermediate between that of light-open and dark-closed stomata. The stomatal apparatus shows two recognizable widths. One (W), termed upper, lies at or near the level of the outer (upper under the microscope) wall of the guard cells, and the other (LW) where its lower wall joins the wall of the flanking epidermal cell. The last columns in table III indicate that the measured differences between the upper widths of open and dark-closed and of wilted and dark-closed stomata are statistically significant.

TABLE III

ANALYSIS OF STOMATAL DIMENSIONS OF THE LEAVES IN TABLE II. STANDARD ERROR $W_1 - W_2$ IS THE STANDARD ERROR OF DIFFERENCE BETWEEN THE RESPECTIVE PAIRS OF WIDTHS. t = (MEAN W_1 − MEAN W_2)/STANDARD ERROR OF DIFFERENCE. A VALUE OF $t > 2$ INDICATES A STATISTICAL SIGNIFICANCE OF 95% OR MORE. A VALUE OF $t < 2$ INDICATES THAT THE DIFFERENCE BETWEEN THE TWO MEANS IS NOT SIGNIFICANT.

Leaf and treatment	Stomatal apparatus					No. of stomata measured	Standard error $W_1 - W_2$	t
	Length μ L	Width μ Upper W	Width μ Lower LW	L/W	W/LW			
V 1	31.3	28.8	21.5	1.90	1.34	12	± 1.0	1.5
2	30.2	25.9	15.4	1.17	1.69	12	± 2.0	2.6
3	34.4	20.7	16.3	1.66	1.26	12		
W 1	31.9	29.9	....	0.94	....	21	± 1.2	3.4
2	29.0	26.8	....	1.08	....	22	± 1.4	4.1
3	32.5	21.0	....	1.55	....	16		
X 1	30.6	26.8	....	1.14	....	10	± 1.6	1.8
2	29.8	23.9	14.6	1.25	1.64	20	± 1.4	2.9
3	32.7	19.8	16.0	1.66	1.25	16		
Y 1	31.7	26.1	....	1.22	....	10	± 1.6	2.3
2	29.5	22.6	....	1.31	....	10		
Z 1	31.8	29.9	....	0.94	....	20	± 1.5	2.0
2	29.1	26.9	....	1.08	....	20		

The differences in upper width between open and wilted stomata are not always significant though the lower widths are very different.

The significant findings may be summarized as follows: (1) The upper width is always greater than the lower width ($L > LW$). (2) The ratio L/W is about the same (1.1) in open and in wilt-closed stomata. In dark-closed stomata it is much higher (1.6). (3) The ratio W/LW is about the same (1.3) in open and dark-closed stomata and is higher (1.65) in wilted stomata. In other words wilt-closed stomata are wide like open stomata at the top and narrow like dark-closed stomata at the bottom. The drawings in figure 4 show the appearance of open, dark-closed and wilt-closed stomata viewed from above, the dotted lines representing the bottoms of the guard

cells. Our conception of a median vertical section through each pair is also represented. The wilted stoma might perhaps be described as "open at the top and closed at the bottom."

The widening of the stomatal apparatus during wilting has commonly been ascribed to reduction of pressure, and at a later stage, to development of tension in the adjoining epidermal cells. Contraction of the outer wall of the epidermis is probably important in Pelargonium, since the upper walls of the guard cells are pulled away from the pore more than the lower ones. Loss of turgor in the guard cells tends to bring their adjacent edges together, but at first this is more than counterbalanced by the opposite pull on the stomatal apparatus. At a later stage, perhaps when contraction of the epi-

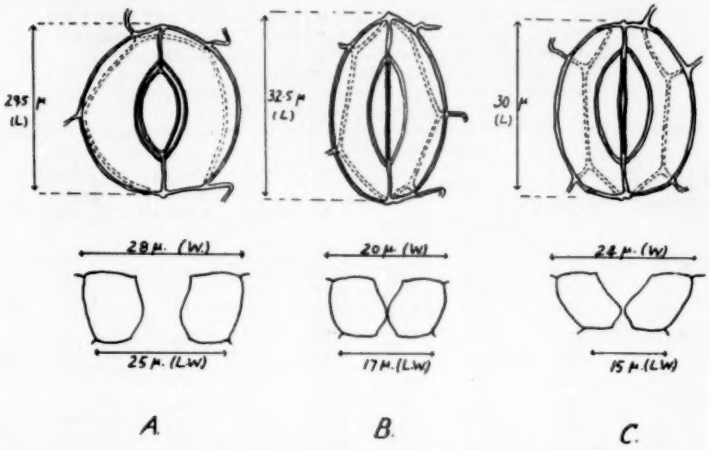

FIG. 4. Diagrams of stomata viewed from above, and in imaginary median vertical section. (A) Open in light; (B) closed in darkness; (C) closed as a result of severe wilting in CO_2-free air.

dermal walls or distortion of the guard cells is approaching its limit, the closing movement predominates. In purely passive closure, however, the tissue tension prevents the edges of the guard cells from becoming closely appressed at the middle of the slit.

The resemblance that wilt-closed stomata bear to light-open stomata is not entirely the result of external tensions. In purely passive closure, by definition, no osmotic change occurs except as the direct effect of the water loss. That is to say, the osmotic pressure at incipient plasmolysis of the guard cells and that of the adjoining cells would remain unchanged, and show the same relative difference as in the light-open state. The condition of passively closed stomata might, therefore, be described as "physiologically open but morphologically shut."

Chloroform

Chloroform vapor was introduced by inserting a 10-cc. graduated cylinder, containing a few cc. of chloroform, into a side arm of the air stream, so that part of the air passed over the surface of the liquid. The rate of evaporation was regulated by varying the amount of flow diverted through this side arm, and was estimated from the reduction in volume of the chloroform. The rate of flow of the whole air stream was kept constant at 80 l./h. Leaves were set up in the leaf chamber under the light and allowed to come to stomatal equilibrium before treatment began. For brief intervals during treatment the leaf chamber was opened for removal of a strip from the epidermis. On each strip 50 to 100 widely scattered stomata were measured. With open stomata, concentrations of chloroform of 0.02 cc./l. and over produced injury and irreversible closure within a few hours. Concentrations below 0.005 cc./l. had no effect. Between these limits the effect varied in different experiments.

In several tests of stomatal behavior alone chloroform produced partial closure that was reversed when the treatment was discontinued. Of the few tests of photosynthesis alone, at least two showed complete stoppage followed by recovery. Only one experiment combining measurement of stomata and photosynthesis was successful in obtaining the critical conditions that seem to be required to produce reversible changes. The results of this experiment are shown in table IV. The details are as follows. Since chloroform vapor absorbs infra-red, the air stream, before entering the infra-red absorption tube, was passed through condensing tubes packed in dry ice, and the condensing system was kept in the circuit both when the leaf chamber was in and when it was cut out for zero determination. Thus any chloroform vapor that remained in the air at or near the temperature of solid carbon dioxide would equally affect the zero and experimental readings on the galvanometer. Stripping of the epidermis was reduced to a minimum so as to damage the leaf as little as possible. It was impossible to use the porometer and be sure that the chloroform dosage was the same inside and outside the cup.

TABLE IV

EFFECT OF CRITICAL DOSAGE OF CHLOROFORM ON PHOTOSYNTHESIS AND STOMATAL APERTURE. LIGHT INTENSITY 1000 fc.

Treatment	Chloroform 0.005 cc/l					No chloroform					
Hours from start	0	¼	½	2	2¼	2½	2¾	3¼	3¾	4½	6½
Photosynthesis (galv. defl.*)	41	22	15	0	0	7	12.5	20	26	40	42
Pore width (μ)	....	....	....	....	1.4	....	....	2.0	....	4.5	6.7

* 50 mm. deflection = approx. 0.075 mgrs. CO_2/liter of air.

Table IV shows the course of photosynthesis during and after chloroform treatment, and the stomatal aperture at critical periods. The initial

width was not measured. If average, it would be about 7.0 μ. The results show that photosynthesis was rapidly inhibited by chloroform and ceased altogether. Pore width declined to about 20% of its maximum. With removal of chloroform, photosynthesis recovered slowly and the stomata reopened still more slowly.

The effect of chloroform in CO_2-free air is shown in table V. The leaf was first brought to equilibrium with light of 100 fc and CO_2-free air, and was then exposed to 0.008 cc. of chloroform per liter of air. During three and one-half hours the decline in stomatal aperture was very slight. Normal air was then supplied and the light intensity raised to counteract its effect on stomatal aperture. Nevertheless a rapid decline in the width of the pores ensued. This movement was gradually reversed when the chloroform was cut out. The whole behavior can be explained on the photosynthetic theory if the chloroform had already incapacitated the photosynthesis

TABLE V

EFFECT OF CHLOROFORM ON STOMATAL APERTURE IN ABSENCE AND PRESENCE OF CARBON DIOXIDE

Hours from start	Chloroform cc/l	Light intensity fc	CO_2 content of the air	Pore width μ
0	0.008	100	zero	7.0
1½	0.008	100	zero	6.6
2½	0.008	100	zero	6.8
3½	0.008	1000	normal	6.5
4	0.008	1000	normal	2.3
4½	zero	1000	normal	1.9
6	zero	1000	normal	3.0
10	zero	1000	normal	4.3
24	zero	1000	normal	5.4
0	0.008	100	zero	6.4
3½	0.002	100	zero	6.3
4	zero	100	zero	2.4
7	zero	100	zero	0.0

mechanism while the air was free of CO_2, but the effect of this on the stomata only became apparent when stomatal opening was made dependent on photosynthesis by return to normal air.

Next, the chloroform concentration was raised from 0.008 to 0.02 cc./l. without changing from CO_2-free air. Again closure ensued, but this time it was found to be irreversible; apparently closure resulting from injury is not prevented by absence of CO_2.

In every case of reversible closure of stomata the closure was incomplete. The greatest degree of closure occurred in the lowest effective concentration of chloroform (0.005 cc./l.), a less degree in slightly higher concentrations (*e.g.*, 0.008 cc./l.), and no distinct reduction of aperture in 0.02 cc./l. within the period during which closure occurred in lower concentrations. It was only after several hours at this concentration that the irreversible type of closure occurred, accompanied by visible signs of injury to

portions of the leaf. To explain this behavior it is suggested that chloroform paralyzes the motility of the guard cells when it has accumulated to a somewhat greater extent than is sufficient to check photosynthesis. The higher the concentration of chloroform, and hence the more rapid its uptake, the less will these two effects be separated in time, until eventually active closure is stopped almost as soon as it has started.

While the complex action of chloroform obviously requires further investigation, the above results indicate that a rather critical dosage of chloroform may produce a type of closing movement that is reversible and can be largely prevented by absence of CO_2.

Photosynthesis in guard cells

We must still consider the question of whether the direct action of light on stomata, as distinguished from its indirect action through photosynthesis in the mesophyll, is also photosynthetic. In the first place, are guard cells capable of photosynthesis? On the one hand, guard cells behave quite differently from chlorenchyma cells as regards accumulation of starch, and it has been argued that their pigment is not chlorophyll (SAYRE, **8**). On the other hand, recent work of ALVIM (**1**) points to the occurrence of a slow rate of photosynthesis in guard cells, and that of FREELAND (**3**) seems to demonstrate that the pigment in one species at least is largely chlorophyll a. CURTIS and CLARK (**2**) cite as the most convincing of Alvim's experiments one in which epidermal strips, taken from leaves that had been starved in darkness until the guard cells were emptied of starch, were floated on water under continuous light. Abundant starch developed in the guard cells of such strips in presence of CO_2 and none in its absence. Whether the comparison is significant depends on how the starch test was made, because, as long as they are in CO_2-free air, even guard cells that have not been starved fail to form starch. But the finding that starch formed in normal air is itself indicative of photosynthesis. In the case of formation of starch in guard cells of intact leaves, Alvim himself postulates that translocation from the mesophyll is a factor. We, therefore, thought it desirable to repeat his experiment in such a way as to preclude photosynthesis in any other tissue of the leaf. L. J. Coulombe assisted the senior author in these few experiments.

A variegated Pelargonium plant was kept in darkness for 11 days, by which time the starch content of its guard cells varied from none at all to mere traces. Some leaves were then covered on both surfaces with black paper, except a portion of the white margin of the leaf, and the plant was placed under the lamp. At intervals the leaf or plant was darkened for two hours before taking strips for starch tests. No starch appeared in guard cells or other tissue covered by the black paper, even after two or three days in the greenhouse. In the exposed margin of the leaf, however, starch was distinctly present in the guard cells after five hours in the light and fairly abundant after two days. Since the condition of the shaded area indicated no apparent translocation to the leaf from other parts of the plant and since none of the green mesophyll of the leaf itself was exposed to light, the con-

clusion is that if, as seems probable, the restoration of starch was primarily due to photosynthesis, the latter must have occurred in the guard cells themselves which are equally pigmented in white and green areas.

Alvim's finding of a gradual recovery of the response of stomata to light, parallel to reformation of starch in the guard cells, was also confirmed, but there was evidence that increase in the store of carbohydrate was not the sole reason for the recovery. From porometer and photosynthesis measurements made on a green leaf that had been starved, it appeared that the extreme meagerness of opening during the first few hours of subsequent exposure to light was partly due to an abnormally low rate of photosynthesis (prolonged induction period) since CO_2-free air added greatly to the opening—not, however, equal to that produced by it in unstarved leaves. It

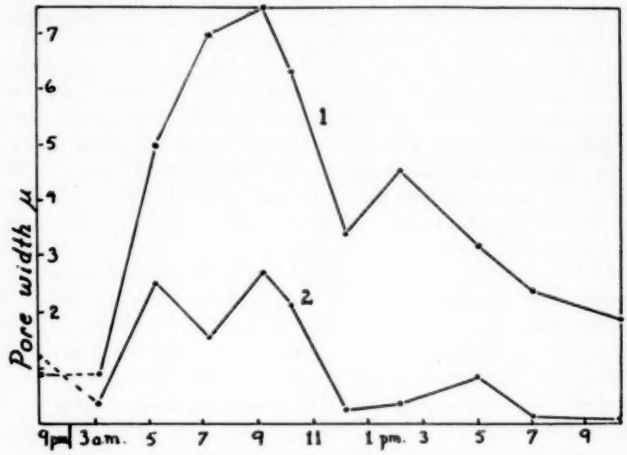

FIG. 5. Time courses of stomatal pore width in green areas (1) and white areas (2) of a variegated leaf during a 24-hour period.

should be mentioned, however, that Alvim did not obtain opening with ammonia treatment at this stage.

Indication that photosynthesis in the guard cells functions in light-induced opening under normal conditions was given by the various results that showed cessation of general photosynthesis to be quickly followed by completion of stomatal closure. The following experiment indicates further that the beginning of inhibition of photosynthesis in the leaf is accompanied by reduction in the effect of light acting directly on the guard cells. The natural daily cycle of stomatal movement was compared for green and white areas of a variegated Pelargonium. The plant was kept outdoors on a bright day in June and strips were taken by Lloyd's method at hourly intervals. Figure 5 shows the results. The point to note in the present connection is

that the respective peaks of opening and also the midday minima were reached simultaneously in the white and green areas. The partial closure was probably due to a mild water deficit, which as we have seen begins to induce closure in green leaves soon after it begins to inhibit photosynthesis. Therefore the synchronization of the response in the two areas supports other evidence that opening in the white area is dependent on photosynthesis in the guard cells.

Discussion

The individual results presented in this paper and the preceding one (**11**), being based on few experiments, are pointers rather than proofs, but they all point in the same direction. Whatever factor other than CO_2 which caused photosynthesis to rise or fall also caused stomata to open or close. Carbon dioxide was the exception that proved the rule. Its complete absence from the air produced about as wide opening in darkness as is usually obtained with light of 400 fc in normal air; and, when photosynthesis was inhibited by other agents than lack of light, absence of CO_2 preserved opening to a similar degree. This action was complicated in the case of wilting by the occurrence of passive closure, and in the case of chloroform treatment by closure due to injury and other effects, but, since these could be separated in time from active closure, the dependence of the latter on accumulation of CO_2 was made evident, at least in the case of wilting. Results obtained with variegated leaves indicate that the main part of the action of light on stomata is indirect, namely through photosynthetic reduction of CO_2 concentration by green mesophyll. Limited evidence pointed to the photosynthetic nature of the direct action of light also.

These statements can be applied only to Pelargonium as far as our results go. There is much evidence in the literature which would support a general extension of the photosynthetic theory, but the antagonistic evidence must also be evaluated. The work of Sierp and Harms was discussed in the first paper (**11**). That of Wilson (**14**) remains to be considered. Regarding the fluctuations in porometer readings with fluctuations in the intensity of sunlight, a minor point is that these curves, especially with Wilson's type of porometer, exaggerate the amplitude of stomatal movement at wide, as compared with narrow, apertures. More important, since the speed of the movements, described as "almost instantaneous," is similar to that shown in our figure 3 in response to sudden changes in v.p.d., it is suggested that the leaves were of the common type that show passive opening with a drop in leaf water balance. This opening may take place without any visible wilting and it occurs when the v.p.d. is raised by increasing the temperature of the leaf as well as by lowering the v.p. of the air. It seems probable, therefore, that the fluctuations in intensity of radiation produced fluctuations in the temperature and rate of transpiration of the leaf tissue.

As regards Wilson's finding that during midday wilting the stomata sometimes continued to open while photosynthesis decreased, and occasionally after it ceased, the following considerations apply: (1) Opening may

have been passive until the condition for active closure was reached. (2) Active closure must have been delayed under the porometer cups because they were not swept by air and the slow stream of air passing through the tissue has to travel a considerable distance in hypostomatous leaves (as those of Camellia and Ligustrum are) and would, therefore, become largely depleted of CO_2 before it reached the cup, even when photosynthesis had been reduced to a low rate. (3) Photosynthesis and porometer measurements were made on different leaves, one in a leaf chamber and one not, so that the times of onset of wilting may have been different in the two. As regards the effect of chloroform treatment, our results differ from Wilson's and also indicate such complexity in the action of chloroform that much further study is needed.

Finally as to the stomatal response to light observed by Wilson in chlorophyll-free leaves, it seems possible that in the case of an etiolated plant exposed to a lamp of 1000 fc passive opening due to wilting may be part, at least, of the explanation. But no such reservation applies to the weak opening reported for a variety of Coleus. Though the finding is contrary to that of PAETZ (**6**) for a chlorophyll-free variety of Caladium, it is in harmony with much of the older work, which found little correlation between width of opening and degree of pigmentation of guard cells situated over chlorophyll-free tissue.

While it seems that plants may differ with regard to the nature of the direct action of light on guard cells, it is probable that its indirect mode of action through the mesophyll is of general occurrence. There are records of response to external CO_2 concentration in a wide variety of plants, and, perhaps, the biological advantage of regulating the supply of CO_2 by the demand for it may justify the expectation that such a mechanism would have a high "survival value."

On the other hand, the specific mode of wilt action as worked out for Pelargonium does not seem to have such general application. In some species a rapid closing movement of stomata is reported to be the first sign of water deficit of the leaf. The rapidity suggests passive movement, which, with a difference in tissue tensions, might be analogous to the delayed closure of Pelargonium. It is also possible, however, that the mechanism of osmotic change in the guard cells of some plants is more sensitive than photosynthesis of the leaf to water deficit. Such a relationship, if it exists, would not imply that these guard cells are insensitive to CO_2.

For hints as to how CO_2 may act on guard cells, we may glance at some of the results of cell physiological studies. Ammonia vapor acting on intact leaves until the guard cells become very slightly alkaline produce about the same degree of opening in the dark as CO_2-free air does (**8, 9, 10**). This and other modes of artificially raising the pH of guard cells result in the same type of intracellular changes as is caused by light (**7**). Light itself causes the pH to rise (**10, 8, 7, 12, 13**). Estimates of the range vary. That of Scarth placed it at from about pH 5.0 in darkness to almost 7.0 in light.

Such a pH change might be brought about by change in CO_2 concentration if the necessary enzymes are present. Later biochemical discoveries of the enzyme phosphorylase and its properties (**4, 5**) and of its presence in guard cells (**15**) lend greater significance to the pH change, because this enzyme promotes *in vitro* the reversible reaction: Starch + inorganic phosphate $\rightleftharpoons$ glucose-1-phosphate and the balance of this reaction depends sensitively on pH within the above range. The ratio of inorganic to ester phosphate drops from 10.8 at pH 5.0 to 3.1 at pH 7.0.

But starch changes, important though they must be usually as a source of osmotic change, are evidently not essential to it. Stomatal movement and starch conversion are not always well correlated, and regular absence of starch, as in Allium, does not prevent normal opening and closure. Artificial change of pH may also induce movement without accompanying starch change (**9**). Speculating on the basis of some indirect evidence we suggest that in addition to utilizing carbohydrate stored within themselves, guard cells may draw on neighboring cells for the material required to increase their osmotic pressure. Polar translocation against the concentration gradient is not infrequent in metabolically active tissues and there is indication that it takes place between the cytoplasm and vacuole of guard cells even when starch–sugar conversion is functioning. The indication comes from the observation that in the course of opening, for example, only the vacuole of the cells shows any marked increase in volume and inferentially in the content of osmotically active substance (see photographs in **9**). The most probable explanation of this behavior would seem to be an active contra-gradient secretion of glucose, etc., from the cytoplasm into the vacuole—a process incidentally which could have much influence on the course of the phosphorylase reaction. The phenomenon of vacuole contraction and the reciprocal expansion displayed by various other types of epidermal cell is also in our opinion best explained by polar transport of solute in one direction or the other across the vacuolar membrane. It seems reasonable to extend the translocation hypothesis to transfer of solute between the guard cells and their neighbors. During stomatal opening, when the osmotic pressure of guard cells rises much above that of other epidermal cells, this movement must be contra-gradient for the most part, so that it cannot properly be ascribed to increased permeability—unless this is defined as of the so-called active type. If photosynthesis occurs in guard cells, escape of sugar at some time is necessary whether there is intake or not, and a controlled translocation to the neighboring cells coincident with stomatal closure would have the greatest biological advantage.

Changes in the osmotic pressure at incipient plasmolysis of subsidiary cells in the opposite direction to those in guard cells have rarely been reported, but the relative volume of the cells is generally such that a transfer of solute sufficient even to double the osmotic pressure of the guard cells would have a scarcely measurable effect on that of the larger cells.

Finally, if active translocation of solute is part of the mechanism of

osmotic change even within the guard cells, the operation of protoplasmic regulation, stressed earlier, is easier to understand, than if the process depended simply on a reversible enzymatic reaction.

Summary

In Pelargonium stomatal closure when induced by mild water deficit or by non-toxic concentrations of chloroform is preceded by inhibition of photosynthesis and may be largely prevented by CO_2-free air. More severe wilting causes passive closure, which is not prevented by absence of CO_2. The guard cells appear to be capable of photosynthesis.

The results, including those presented in a previous paper, confirm that the effect of light on stomatal opening depends on photosynthesis and, mainly at least, on reduction of CO_2 concentration in the leaf.

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AN OUTLINE OF ENERGETICS IN RELATION TO THE MOVEMENT OF MATERIALS THROUGH A TWO-PHASED SOLUTION SYSTEM

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Introduction

As an introduction to discussion on certain theoretical considerations of modes of expression and factors possibly concerned in the movement of materials through a 2-phased solution system, it is essential to outline the thermodynamic principles thus involved. This thermodynamic approach to the energetics of the process will be followed by the considerations referred to above (**4**). General reference should be made to Lewis and Randall (**6**), to Eastman and Rollefson (**5**), and to detailed treatises made earlier on the subject of solute and solvent transfer (**1, 2, 3**). This outline extends considerations on the influence of solute in solutions, in particular. Other influences are effective in the net movements of materials in nature. These latter have been discussed elsewhere (**1, 2, 3**) from the energetics' viewpoint and their importance is evaluated, where possible, in an article to follow (**4**).

Outline of solute effects in solution

For simplicity, as before (**1, 2, 3**), the movement of either solute or water may be discussed under rather ideal conditions, through a 2-phased aqueous solution system where a semi- or differentially permeable membrane is interposed as an interfacial boundary. The fundamental relationship between the tendency for movement of either component of solution is given by

$$(\bar{f}_e - \bar{f}_i) = RT \ln (a_e) - RT \ln (a_i)$$

or

$$(\bar{f}_e - \bar{f}_i) = RT \ln \left(\frac{a_e}{a_i}\right), \tag{1}$$

in which $(\bar{f}_e - \bar{f}_i)$ is the net escaping tendency expressed as the partial molal free energy difference [1] between an external phase e and an internal phase i, in ergs per mole; R is the gas law constant, in ergs per mole per degree; T is the absolute temperature of the system; and the ratio $\frac{a_e}{a_i}$ is the molal activity (corrected molal composition in moles per kilogram of solvent) ratio of the constituent component in the two phases.

For certain purposes, the partial molal free energy differences of either component may be expressed as another energy intensity, namely the partial molal free energy difference per liter of component flux which has been termed the volumed specific free energy difference. These general relation-

[1] This measures the energy for useful work of migration rather than the total work (**2**).

ships between the specific free energies and the partial molal free energies are given by

$$Fs = \frac{\pm}{\mp}(p - p_o) = \frac{\pm k\,(\bar{f} - \bar{f}^o)}{\bar{v}} \tag{2}$$

and

$$NIF = \Delta Fs = (Fs_e - Fs_i) = \frac{\pm}{\mp}(p_e - p_i) = \frac{\pm k\,(\bar{f}_e - \bar{f}_i)}{\bar{v}} \tag{3}$$

in which NIF is the net influx specific free energy (net influx intensity), in atmospheres; ΔFs for either the solvent (subscript 1) or the solute (subscript 2) component is the specific free energy (volumed) difference due to the presence of solute in solution, in liter atmospheres per liter of constituent component migrating; Δp is the pressure difference necessary to make $\bar{f}_e$ equal to $\bar{f}_i$, in atmospheres; k is the conversion factor from ergs to liter atmospheres, viz., 9.87×10^{-10} [equivalent to $1/1.013 \times 10^9$, as used earlier (**2, 3**)]; $\bar{v}$ is the partial molal volume of a constituent component in solution in liters; and other notations are as before.

Considering first the movement of solute, the resultant energy intensity expressed as a volumed specific free energy between two contiguous phases differing only in solution composition is given by

$$\Delta Fs_2 = k_a \ln \frac{(a_e)_2}{(a_i)_2}, \tag{4}$$

in which k_a is a characteristic coefficient; $\frac{a_e}{a_i}$ is the ratio of the effective molal compositions (activities) of solute in the two phases e and i respectively; and ΔFs_2 is the resultant specific free energy in atmospheres, *i.e.*,

$$\Delta Fs_2 = \frac{(\bar{f}_e - \bar{f}_i)_2}{\bar{v}_2}, \tag{5}$$

in which $(\bar{f}_e - \bar{f}_i)_2$ is expressed in liter atmospheres per mole; and $\bar{v}_2$, in liters. The proportionality coefficient k_a in equation 4 is given by

$$k_a = \frac{\Delta Fs_2}{\ln \frac{(a_e)_2}{(a_i)_2}} = \frac{RT}{\bar{v}_2}, \tag{6}$$

in which R is the gas law constant, in liter atmospheres per mole per degree; T is the absolute temperature; and $\bar{v}_2$ is the partial molal volume of the constituent solute in solution, in liters.

Where the flux is limited to effects arising only from a solution composition difference between phases, another relationship is frequently employed, which may be recognized as Fick's law of diffusion (**5**). This is given by

$$P_2 = (\Delta p)_2 = k_b\,(C_e - C_i)_2, \tag{7}$$

in which P_2 is the net diffusion "pressure" of a constituent solute species in solution, in atmospheres; k_b is a characteristic coefficient; $C_e - C_i$ is the concentration difference of the solute species between phases e and i, expressed in grams per liter, and other notations are as before. The proportionality coefficient is given by

$$k_b = \frac{P_2}{C_e - C_i} = \frac{\frac{RT}{\bar{v}} \ln \frac{a_e}{a_i}}{C_e - C_i} = \frac{RT}{\bar{v}} \ln \frac{a_e}{a_i} \times \frac{1}{C_e - C_i}, \tag{8}$$

in which the factors are the same as in earlier equations. It should be stressed that P_2 is completely equivalent to a volumed specific free energy ΔF_s, expressed in dimensions of liter atmospheres per liter of solute flux. Although expressed in dimensions equivalent to pressure, the energy intensity is strictly not a physical pressure. However, the flux intensity so expressed may be evaluated through measurement of the pressure difference $(p_e - p_i)$ on two contiguous phases, necessary to equilibrate the rates of flux across the interfacial boundary.

Equations 6 and 8 are interrelated by the relation

$$k_a = \frac{k_b C_e - C_i}{\ln a_e/a_i}, \tag{9}$$

and

$$k_b = \frac{k_a \ln a_e/a_i}{C_e - C_i} = \frac{\frac{RT}{\bar{v}} \ln a_e/a_i}{C_e - C_i}. \tag{10}$$

The net rates of flux corresponding to the energy intensity differences expressed in equations 4 and 7 are given by the differentials

$$\frac{dm}{dt} = A\, k_a\, k_d \frac{\ln a_e/a_i}{x}, \tag{11}$$

and

$$\frac{dm}{lt} = A\, k_b\, k_d \frac{(C_e - C_i)}{x}, \tag{12}$$

in which dm is the differential mass moving in differential unit time, dt; A is the effective area for movement through the interface; x is the distance along the axis of flux; and other factors are as before.

Strictly speaking, the rate of net influx (RNI) of solute from phase e to phase i is equal to a flux coefficient times the energy intensity (EI) gradient across the interposed membrane or boundary. Here, for means of comparison between energy intensity gradients and concentration gradients in net movement of materials, it is assumed that the net rate of flux is proportional to the energy intensity gradient. Qualifications in such an assumption are recognized (cf., for example, (**6**), pgs. 160 and 584). In general terms, the rate of net influx is given by

$$RNI_z = k_\phi \times \frac{EI}{L} \tag{13}$$

and expressed as the net mass moving across a unit area of interface in unit time. The dimensions are $\frac{m}{L^2t}$.

The flux coefficient k_ϕ is defined to equal the net mass of a constituent component moving per unit time per unit area of boundary surface per unit energy intensity gradient. From the differential equation 11, this coefficient is given by the following dimensions

$$k_\phi = k_p \times \frac{m}{t} \times \frac{1}{L^2} \times \frac{1}{(EI/L)} \tag{14}$$

$$k_\phi = k_p \times \frac{m}{tL^2} \times \frac{L}{\frac{mL^2/t^2}{\bar{v}}} = \frac{m}{tL^2} \times \frac{L}{\frac{mL^2/t^2}{L^3}} = t. \tag{15}$$

In equation 14, k_p is a property of the interface, modifying the flux rate with respect to a reference free movement across the boundary. This relative permeability coefficient is without dimension in equation 15.

The flux coefficient k_ϕ is often associated with the diffusion coefficient k_d as derived from Fick's law (see equations 7 and 12). The latter coefficient is related to a concentration gradient rather than to an energy intensity gradient as expressed in equations 14 and 15. The diffusion coefficient is defined to be the mass moving, under similar conditions to those discussed previously, per unit concentration gradient, where the concentration is expressed in grams per liter for dimensional accuracy. Dimensionally, the diffusion coefficient is given by

$$k_d = k_p \times \frac{m}{t} \times \frac{1}{L^2} \times \frac{1}{\frac{m}{L^3} \times \frac{1}{L}} = \frac{L^2}{t}. \tag{16}$$

The two coefficients, the flux coefficient k_ϕ and the diffusion coefficient k_d, are equated through the relationship

$$k_\phi = k_d \times \frac{1}{k_b}. \tag{17}$$

Dimensionally the two coefficients are related as follows:

$$t = \frac{L^2}{t} \times \frac{1}{\frac{RT}{\bar{v}} \ln \frac{a_e}{a_i} \times \frac{1}{C_e - C_i}} \tag{18}$$

or

$$t = \frac{L^2}{t} \times \frac{t^2\,T\,L^3}{m\,L^2\,T} \times \frac{m}{L^3} = t. \tag{19}$$

The rate of net influx was given in general terms in equation 13. More completely, it may be expressed by

$$RNI_s = k_\phi \times \frac{EI}{L} = k_\phi \times \frac{\Delta Fs_2}{L} \tag{20}$$

and in dimensions of

$$RNI_s = t \times \frac{\dfrac{mL^2}{t^2} \Big/ \bar{v}}{L} = t \times \frac{\dfrac{mL^3}{t^2}}{LL^3} = \frac{m}{L^2 t}. \tag{21}$$

From the relationship expressed in equation 17, equation 21 may be reset as follows:

$$RNI_s = k_\phi \times \frac{EI}{L} = k_d \times \left[\frac{EI}{L} \times \frac{1}{k_b}\right]. \tag{22}$$

The values

$$\left[\frac{EI}{L} \times \frac{1}{k_b}\right] = \left[\frac{EI}{L} \times \frac{1}{\dfrac{RT}{\bar{v}} \ln \dfrac{a_e}{a_i} \times \dfrac{1}{C_e - C_i}}\right]$$

have dimensions

$$\left[\frac{m}{Lt^2 L} \times \frac{t^2 T}{m L^2 T} \times \frac{L^3}{1} \times \frac{m}{L^3}\right] = \left[\frac{m}{L^3 L}\right], \tag{23}$$

where $\frac{m}{L^3 L}$ may be recognized as the concentration gradient $\frac{C_e - C_i}{L}$. The rate of net influx then becomes

$$RNI_s = k_d \times \left[\frac{m}{L^3} \times \frac{1}{L}\right] = \frac{L^2}{t} \times \frac{m}{L^3} \times \frac{1}{L} = \frac{m}{L^2 t}. \tag{24}$$

In words, here the rate of net influx equals the diffusion coefficient times a concentration (expressed in grams per liter) gradient.

Since the rates of net influx in equations 21 and 24 have identical dimensions and equal magnitudes, under similar conditions, the other factors in these relations can be equated and evaluated. Such computed values, for certain assumed ideal conditions, are assembled in table I. The data of table I were derived as follows: First, arbitrary values for the molar solution concentrations c_e and c_i were assigned in all cases but number 3. These latter quantities (case 3) were derived from the relationship

$$\Delta Fs_2 = \frac{(\bar{f}_e - \bar{f}_i)}{\bar{v}}$$

where

$$(\bar{f}_e - \bar{f}_i) = RT \ln \frac{a_e}{a_i}, \tag{25}$$

assigning a value of unity to ΔFs_2. Second, the diffusion coefficient k_d was assumed to be constant. Third, the rates of net influx were computed from

equation 24 using assigned concentration differences. These net influx tendencies are equal on the concentration and on the specific free energy bases. Fourth, energy intensity values were computed by means of equation 6. In case 3, ΔFs_2 was set at unity, and particular values of c_e and c_i derived by means of equation 6, assuming first, that the molality is equal to the activity (*i.e.*, the activity coefficient equals one), and second, that the molarity approximates the molality (*i.e.*, the solution density approaches unity). The energy intensities are expressed, per unit gradient, in column 10. Fifth, the flux coefficients k_ϕ were computed by means of equation 13.

One further set of data was computed, *viz.*, the theoretical rate of energy expenditure (REE). The actual rate will of course depend on the efficiency of the process of energy supply to the movement. These energy expenditure rates were derived from the relationship

$$REE = RNI \times \Delta Fs_2 \times A \times \frac{\bar{v}}{m}. \tag{26}$$

The dimensions are

$$REE = \frac{m}{Lt^2} \times \frac{m}{L^2t} \times \frac{L^2}{l} \times \frac{L^3}{m} = \frac{mL^2}{t^2} \times \frac{l}{t}. \tag{27}$$

In equation 26, A is the area of flux and m is the molecular weight of the solute (74.55 gm. per mole for KCl). The ratio $\frac{\bar{v}}{m}$ in equation 26 is included here, since ΔFs_2 is expressed in liter atmospheres per liter of solute flux and RNI is in grams. The rate of energy expenditure is thus expressed in dimensions of liter atmospheres per second.

The data of table I show several interesting features. Where the diffusion coefficient k_d is considered constant, it may be seen that the flux coefficient k_ϕ varies with the relative concentrations of the aqueous media of the two phases. Case 1 shows that where the compositions of the two media are equal, the concentration and energy intensity gradients are zero and thus the rates of net influx and energy expenditure are zero. Case 2 shows that where the ratio of the compositions of the two media is infinite, the energy intensity gradient and rate of energy expediture theoretically approach infinity. At the same time, a finite rate of net influx may be computed on the concentration basis (cf. (7), fig. 30), even though the mathematical solution, computed from the equation derived from the energy intensity gradient, is not defined. Case 3 shows the computed values where the energy intensity is set at unity and an arbitrary value of internal concentration is taken. Case 4 compared with case 5 shows that where the diffusion coefficient of the solute is considered constant, change in the relative compositions of the two media such that this difference is constant, leads to an inverse variation in the energy intensity and coefficient of flux, such that the rates of net flux are equal. Since in the particular cases cited, the energy intensity increases from condition 4 to 5, the energy expenditure rate must of necessity increase likewise. Case 5 compared with case 6, shows that where

TABLE I

RELATIONSHIPS AMONG ENERGY INTENSITY AND CONCENTRATION DIFFERENCES, AND RATES OF NET FLUX AND ENERGY EXPENDITURE.

	1	2	3	4	5	6	7	8
	Solution	Concns.	Solution	Concns.		ΔC	k_d	k_ϕ
	c_e	c_i	C_e	C_i	$\frac{a_e}{a_i}$	$(C_e - C_i)$		
	n/L^3	n/L^3	m/L^3	m/L^3		m/L^3	L^2/t	t
	mols per liter	mols per liter	gm. per liter	gm. per liter		gm. per liter		
1	.001	.001	.075	.075	1	0	1.5×10^{-9}**	**
2	.001	0	.075	0	∞	.075	"	~ 0
3	$\sim$.001001*	.001	$\sim$.0746	.0745	1.0008	$\sim$.0001	"	1.50×10^{-9}
4	.003	.002	.225	.150	1.50	.075	"	2.26×10^{-9}
5	.002	.001	.150	.075	2.00	.075	"	1.33×10^{-9}
6	.004	.002	.300	.150	2.00	.150	"	2.66×10^{-9}

	9	10	11	12	13
	Concn. gradient $(C_e - C_i)/L$	Energy intensity gradient $(\Delta Fs_2/L)$	Rate net influx (Concn. basis)	Rate net influx (Spec. free energy basis)	Rate energy expenditure
	m/L^3L	m/Lt^2L	m/L^2t	m/L^2t	mL^2/t^2t
	gm. per liter per cm.	Atm. per cm.	gm. per sq. cm. per sec.	gm. per sq. cm. per sec.	Liter atm. per sec.
1	0	0	0	0	0
2	.075	∞	1.125×10^{-6}	1.125×10^{-6}†	∞
3	.0001	1	1.500×10^{-9}	1.500×10^{-9}	4.02×10^{-13}
4	.075	4.95×10^{2}	1.125×10^{-6}	1.125×10^{-6}	1.49×10^{-7}
5	.075	8.47×10^{2}	1.125×10^{-6}	1.125×10^{-6}	2.55×10^{-7}
6††	.150	8.47×10^{2}	2.250×10^{-6}	2.250×10^{-6}	5.10×10^{-7}

*$\sim$ indicates approximately; the approximate value of c_e in column 1 and the approximate ratio in column 5 were computed (assuming the activity coefficients and solution densities respectively equal to unity) from setting ΔFs in column 10 equal to unity in the relation

$$\Delta Fs_i = \frac{RT}{\bar{v}} \ln \frac{a_e}{a_i}, \text{ as follows:}$$

$$\Delta Fs_i = \frac{2.303 \times 0.0821 \times 298}{0.02} \log \frac{a_e}{a_i} = 2.815 \times 10^3 \log \frac{a_e}{a_i}$$

$$\log \frac{a_e}{a_i} = \frac{1}{2.815 \times 10^3} = 3.55 \times 10^{-4}, \text{ therefore}$$

$\frac{a_e}{a_i}$ = antilog $3.55 \times 10^{-4} = 1.0008$. Then setting $c_i = 0.001$ molar, and substituting c_e and c_i for a_e and a_i respectively,

$$c_e = \frac{1.0008}{c_i} = \frac{1.0008}{0.001} = 0.0010008 \text{ or } \cong 0.001001 \text{ molar.}$$

**At the kinetic steady state between the two media, k_d is of the same magnitude as that where differences in concentration exist; k_ϕ under similar conditions is indeterminate.

†The rate of net influx on the specific free energy basis is practically equal to that on the concentration basis, viz., 1.125×10^{-6} gm. per sq. cm. per second. Since the energy intensity gradient approaches infinity at $t = 0$, the flux coefficient (k_ϕ) approaches zero.

††For all cases (1 to 6), k_c is a constant, being equal to $RT = 24.45$; k_a is approximately constant, being equal to $\frac{RT}{\bar{v}} = 1.223 \times 10^3$. k_b is variable and may be computed by equation 8 or 10; in case number 6, $k_b = 5.63 \times 10^3$. See text for the nature of these factors.

§In the computations, the interfacial thickness L is set at unity.

the diffusion coefficient of the solute is considered constant, change in the relative compositions of the two media such that the ratio of the compositions and thus the energy intensity is constant, leads to a parallel variation in the concentration gradient and coefficient of flux, such that the rates of net flux are equal. Since in the particular cases cited, the rate of net flux increases from condition 5 to 6, the energy expenditure rate must of necessity increase likewise.

Analogous reasoning may be applied to the movement of water through the 2-phased system. Here, the net flux tendency of the solvent component may be expressed in two fundamental equations similar to those for a constituent solute component. Thus, where subscript 1 refers to the solvent,

$$NIF = \Delta Fs_1 = \bar{\mp} (p_e - p_i)_1 = \frac{\pm (\bar{f}_e - \bar{f}_i)_1}{\bar{v}_1}, \quad (28)$$

and

$$\Delta Fs_1 = k_a \ln \frac{(a_e)_1}{(a_i)_1} = \frac{RT}{\bar{v}_1} \ln \frac{(a_e)_1}{(a_i)_1}. \quad (29)$$

Here, k_a is equal to $\frac{RT}{\bar{v}_1}$.

Similar to the treatment of solute flux, water flux may be expressed by

$$P_1 = (\Delta p)_1 = k_b (C_e - C_i)_1, \quad (30)$$

in which k_b bears a similar relation to k_a as before.

In the special case of water flux tendency, an additional relationship holds approximately, at sufficiently dilute solute concentration in solution, which may be recognized to be the van't Hoff equation, *viz.*,

$$\Delta Fs_1 = P_1 = -(p_e - p_i)_1 = -k_c (k_v c_e - k_v c_i)_2 = -RT (k_v c_e - k_v c_i)_2, \quad (31)$$

in which $k_c = RT$, and c_e and c_i are the molar concentrations of the external and internal media respectively, expressed in moles per liter, for dimensional accuracy. The formal resemblance between equations 30 and 31 may be noted. However, k_b in equation 30 is not equal to k_c in equation 31. Further, in equation 30, the concentration difference refers to that of the water component, while in equation 31, the concentration difference refers to that of the solute component in solution in the two phases. The factor k_v in equation 31 is the well known van't Hoff coefficient.

The treatment of rates of net influx and energy expenditure for water may be carried forward in a manner similar to that for the solute, where appropriate diffusion and flux coefficients are applied. Further, the factor $\bar{v}_1$ in this case refers to the partial molal volume of the solvent component, whereas in the treatment for solute it referred to the partial molal volume of the constituent solute species.

Reference was made earlier to the nature of the value P in equation 7. Likewise in equations 30 and 31 this net diffusion "pressure" should be considered to be an energy intensity of specific (volumed) nature and not a real pressure, even though expressed in such dimensions. Where expressed

in words as a "pressure" this limitation should be recognized. Where it refers to solute flux tendencies, it has been frequently termed the net diffusion pressure of solute, and where it refers to solvent flux tendencies, it has been termed net diffusion pressure of water, in earlier literature. Where restricted to diffusion in the strict sense of Fick, the values of P and ΔFs for a constituent component are synonymous, differing only in phraseology.

WASHBURN (7) has considered the diffusion "pressure" of solute (P_{g2}) to be related to thermal effects and attractions between particles of the system. For comparison, analysis of these relations is given by

$$P_{g2} = P_{T2} + P'_a - (P_a + P_b), \tag{32}$$

using notations of WASHBURN (7, pp. 189–198). The above is his equation 30. In it P_a and P_b are the interionic attractions (in pressure dimensions) exerted between the water and solute, and among the solute molecules respectively in the solution phase; and P'_a is the interionic attraction exerted between the water molecules on the other side (water phase) of the interposed membrane and solute in the solution phase. Under ideal conditions, the diffusion pressure P_{g2} for solute, equals the thermal pressure (P_{T2}), or in the limit may approximate to a hypothetical "kinetic pressure" in solution, based on derivations from the gas law (note subscript g). This last, "kinetic pressure," may be given by

$$P_{g2} = RT\,(c_e - c_i)_2 \tag{33}$$

He states, "The measurement of the rate of diffusion of the solute in a given solution is, in fact, the only safe and certain method for determining its diffusion pressure." Assuming therefore, that the rates of net influx computed in table I apply in calculations related to the net influx intensity and diffusion "pressure," as well as this "kinetic pressure," we have

$$RNI = k_\phi \times \frac{EI}{L}, \tag{13}$$

$$RNI = k_d \times \frac{(C_e - C_i)_2}{L}, \tag{24}$$

and

$$RNI = k_g \times \frac{(c_e - c_i)_2}{L}. \tag{34}$$

From equations 24 and 34,

$$k_g = k_d \frac{(C_e - C_i)_2}{(c_e - c_i)_2}, \tag{35}$$

and in dimensions,

$$k_g = \frac{L^2}{t} \times \frac{m/L^3}{n/L^3} = \frac{mL^2}{nt}. \tag{36}$$

In case 6 of table I, for example, k_g may be computed by means of equation 35 as follows:

$$k_g = 1.5 \times 10^{-5} \times \frac{0.150}{0.002} = 1.125 \times 10^{-3}.$$

Further, P_{g2} in case 6, may be computed by means of equation 33 as follows:

$$P_{g2} = k_c (c_e - c_i) = RT (c_e - c_i)_2 = 4.90 \times 10^{-2} \text{ atmospheres},$$

where $k_c = RT$.

P_2 of equation 7 is related to P_{g2} of equation 33 as follows:

$$P_2 : P_{g2} = k_b (C_e - C_i)_2 : k_c (c_e - c_i)_2. \tag{37}$$

In case number 6, these factors are quantitatively related as follows:

$$P_2 = \frac{P_{g2} \times k_b (C_e - C_i)_2}{k_c (c_e - c_i)_2} = \frac{4.90 \times 10^{-2} \times 5.63 \times 10^3 \times 0.150}{0.0821 \times 298 \times 0.002} = 8.47 \times 10^2.$$

Particular attention should be drawn to the computed magnitudes of P_{g2} and P_2 for case 6. Obviously, direct use of derivations from perfect gas law relations, to migration of solute in solution systems, even if applicable thereto, can at best but lead to an incomplete analysis of the factors involved. Further, indirect computation of water flux tendencies based on "perfect gas pressures" of solute, would seem to be related merely through formal resemblance in equational form rather than to any basis in fact (compare equations 31 and 33).

This outline has been restricted to influences due to the presence of solute in solution where the relative concentrations in the two phases differ. Other influences such as pressure, temperature, galvanic, and metabolic differences across the interposed boundary are usually involved. The energetics of these latter have been considered earlier (**1, 2, 3**) and an approach toward their evaluation in nature is made elsewhere (**4**). Where other influences are involved, the net specific free energies of either component related to solute effects in solution (synonymous only here with net diffusion "pressure") are but a part of a resultant of all the factors concerned.

Other effects in solution

On the specific free energy basis, the net influx intensity for either solute or solvent, for any particular influence is given by

$$(F_e - F_i) = \frac{(\bar{f}_e - \bar{f}_i)}{\bar{v}} = \frac{RT \ln \frac{a_e}{a_i}}{\bar{v}}. \tag{38}$$

In any particular 2-phased system in nature, usually more than one influence is effective at any time, *i.e.*, two or more influences may be acting simultaneously to modify the free energy of the components. Besides the influence of solute in solution *per se*, temperature and pressure differences and galvanic effects may come into play.

Where other influences are simultaneously involved, the net influx tend-

ency for either component is equal to the difference between the sums of those specific free energies causing the constituent component to tend to move inward (influx) and those causing a tendency toward outward movement (efflux). The net influx specific free energy is then given by

$$NIF = \Sigma IF - \Sigma EF. \tag{39}$$

For either solute or solvent the net influx intensity is given in more detail by

$$NIF = (\Sigma F_{(-\Delta f)_i} + \Sigma F_{(\Delta f)_e}) - (\Sigma F_{(-\Delta f)_e} + \Sigma F_{(\Delta f)_i}). \tag{40}$$

in which the subscripts (Δf) and ($-\Delta f$) refer to influences in or on a phase of the system, which increase or decrease respectively, the partial molal free energy of a constituent component of a solution from that in a reference state to that in a given state (**1, 2, 3**).

It should be noted, that the considerations here posed relate to conditions at any particular time t. With the pasage of time, concurrent alterations in the energetic relationships of flux of both solute and solvent obtain. The interrelated conditions of movement of both components are continuously dynamic. Actual data on energy expenditure in the processes of solute or water flux must in the ultimate analysis be based on the integrated effects of time.

Summary

A concise equational analysis of the process of movement of solute and water through a 2-phased solution system is presented. This particularly discusses the effects due to the presence of solute in solution. Comparison of discussions relating diffusional movement based on concentration differences with that based on free energies is made.

This survey points up two situations, first, where concentration differences between phases alone are concerned, a constituent component of solution will tend to move with the direction in which the concentration (or diffusion "pressure") of the species in question, decreases. Second, where other factors such as temperature, pressure, or metabolism are or are not concerned also, a constituent component of solution will tend to move through a 2-phased system with the direction in which the specific free energy of the species in question, decreases. In other words, under all conditions, the latter situation obtains.

Since movements in natural systems include flux both with and against the direction in which the concentration of a particular constituent component in solution decreases, an energy intensity approach is to be preferred. It is necessary in any case where energy expenditures are to be evaluated, and where influences other than the mere presence of solute in solution are involved, as is general in nature.

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PHYTOTOXICITY OF IMIDAZOLINE DERIVATIVES AND RELATED COMPOUNDS

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(WITH FOUR FIGURES)

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Introduction

In recent years many types of chemicals have been tested for activity in promoting or inhibiting growth of plants. The present paper reports on phytotoxic effects and on relationships between chemical structure and activity of imidazoline derivatives and related compounds, oxazolines, etc., some of which are extremely toxic to higher plants.

Certain imidazoline derivatives and related substances such as benzedrine and adrenaline have long been known to possess heart-stimulating activity. HARTMAN and ISLER (**2**) reported that 2-benzyl imidazoline was highly effective as a heart stimulant, but that its activity was eliminated by replacing the benzyl group with aliphatic chains with the exception of the chains containing six to eight carbon atoms. More recently BELLMONT and MEIER (**1**) showed that certain imidazolines were 10 to 100 times more effective than adrenaline.

As regards physiological effects of imidazolines on plants, these compounds were selected for testing in 1947 solely on the basis of their structural relationship to a substituted benzyl imidazolidinethione, which we had found to possess certain growth-regulatory properties. However, WELLMAN and MCCALLAN (**11**) and THURSTON *et al.* (**7**) have studied the fungicidal action of substituted 2-heptadecyl imidazolines and noted that toxicity to plants increased as the chain length in 2-position was shortened to C_{13} and C_{11}. Furthermore, toxicity was increased by lengthening the chain in 1-position to C_6, whereas the toxicity was markedly decreased by either complete elimination of this chain or by the introduction of a polar group onto the molecule.

Material and methods

The chemicals used are listed in part in table I and will be further specified in connection with the results. All compounds were tested first for their effects on the growth of germinating seeds, by a technique similar to that of SWANSON (**6**). Seeds of wheat (*Triticum vulgare* Vill., variety Henry) and radish (*Raphanus sativus* L., variety Scarlet Globe) were germinated on filter paper in Petri plates moistened with 5 cc. of a water solution or suspension of the compound under observation. Ten seeds were used in each plate and all treatments were duplicated throughout a six-step concentration range from 0 to 500 p.p.m. After four days of growth in an air-conditioned

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dark room maintained at 24° C and about 88% relative humidity, the seedlings were removed and the shoots and primary roots were measured. Statistical analysis of several typical results indicated that variations up to 20% might occur in a given experiment and all interpretations of results are made accordingly.

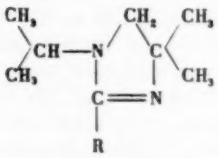
Compounds which proved to be toxic in the above tests, or were of interest because of their similarity to more active compounds, were further tested for their effects on the growth of larger plants in the greenhouse. These tests included spray applications to the aerial parts of the plants and addition of solutions to the soil in which the plants were grown. All spray tests were made with sufficient volumes to completely wet the foliage and all soil tests were made with enough solution to saturate the soil in the pots.

1-isopropyl-2-nonyl-4,4-dimethyl-2-imidazoline, which was highly toxic in all these tests, was used in respiration experiments. The conventional Warburg technique was employed. Discs of fresh leaf tissue were suspended in phosphate buffer of pH 7.0 and imidazoline was added from the side arm.

Finally, the two most toxic imidazolines were utilized in the field for control of weeds in corn and soybean plots. Further details of experimental procedures will be given in connection with the results.

Results

IMIDAZOLINES.—Compounds tested were 1-isopropyl-4,4-dimethyl-2-imidazolines of the following type:



The R-group in 2-position represents alkyl groups 1 to 17 carbon atoms long. The results obtained with ten of these compounds, all used at a concentration of 100 p.p.m., in the germination test, are summarized in figure 1. It may be seen that compounds with R-groups shorter than C_7 were only slightly toxic. Increases in the length of the R-group to C_7, C_9, and C_{11} greatly enhanced toxicity, while a further increase to C_{17} caused a marked reduction in toxicity. However, unsaturation of C_{17} chains restored toxicity to a level comparable with that of the most active members of the series. With higher than 100 p.p.m. concentrations of compounds whose R-groups were C_7 or longer seedling inhibition was complete. Moreover, compounds with R-groups of five or less C atoms produced lower than 50% inhibition, even at 500 p.p.m. The rather specific chain length requirement for high toxicity is strikingly illustrated by the 10-fold increase in toxicity caused by the substitution of the C_7 for the C_5 alkyl group.

The same 10 compounds were used in spray experiments with six-inch tomato plants. Six days after treatment, all plants were harvested and the

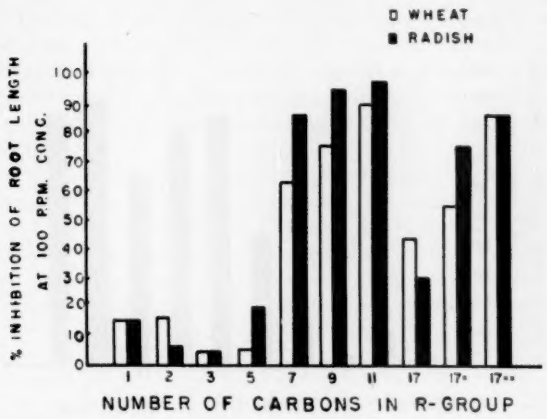
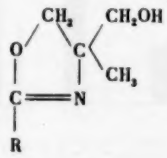


FIG. 1. Seedling response to 10 1-isopropyl-4,4-dimethyl-2-imidazolines differing only in the nature of the R-group at 2-position. (=) double bond in R-group at 8-position. (==) double bonds in R-group at 8,11-positions.

aerial parts weighed. The differences in fresh weight of control and sprayed plants were used as a measure of toxicity. The results obtained with 0.06% spray concentrations, summarized in figure 2, show effects and relationships between structure and activity similar to those obtained in the germination tests. Compounds with alkyl groups of seven or more C atoms either killed or did extreme damage in concentrations as low as 0.06%, while compounds with R-groups of five or less C atoms, even in 1% concentration, produced no measurable toxic effects.

Plants treated with the most active compounds lost turgidity within one hour and large areas of nearly colorless tissue developed on their leaves. The plants dried so quickly that death ensued within 48 hours. Plants treated with non-lethal concentrations usually recovered slowly from severe tissue dehydration which was accompanied by moderate to severe leaf abscission. Only very slight formative effects were observed.

OXAZOLINES.—To obtain more information on the relationship between chemical structure and toxicity, several oxazolines of the following type were compared in the germination test:



The R-group in 2-position represents alkyl groups of from five to 17 carbon atoms.

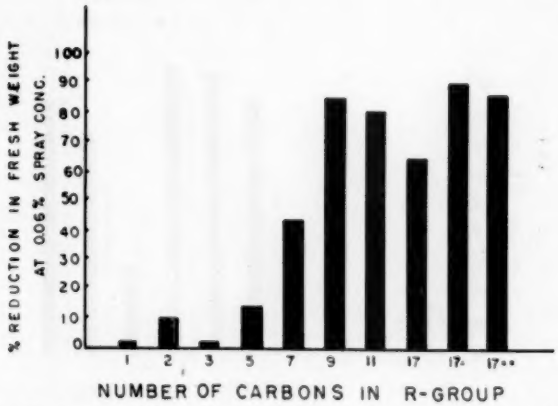


FIG. 2. Fresh weights of tomato plants harvested six days after spraying with 10 1-isopropyl-4,4-dimethyl-2-imidazolines differing only in the nature of the R-group at 2-position. (=) double bond in R-group at 8-position. (==) double bonds in R-group at 8,11-positions.

The results obtained at 100 p.p.m. concentration are summarized in figure 3. All oxazolines tested were much less toxic than imidazolines with corresponding R-groups and the peak was apparently reached with an R-group two carbons shorter than in the imidazoline series.

All oxazolines of this series contained a hydroxyl group in the 4-position which might account for the low toxicity of these compounds. However,

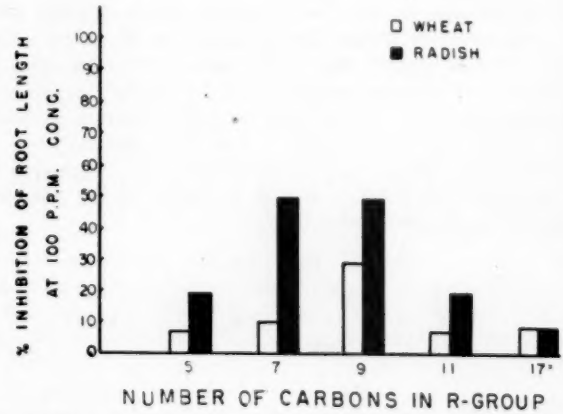


FIG. 3. Seedling response to five 4-methyl-4-hydroxymethyl-2-oxazolines differing only in the nature of the R-group at 2-position. (=) double bond in R-group at 8-position.

2-undecyl-4,4-dimethyl-2-oxazoline was also tested and found to be somewhat more toxic than oxazolines of the above series, but much less toxic than comparable imidazolines. It was concluded therefore that the nature of the ring also contributed to the toxic properties of the imidazoline derivatives. None of the oxazolines was considered sufficiently toxic to warrant greenhouse tests.

OTHER COMPOUNDS.—Several compounds structurally related to imidazolines were compared in the germination test. The results obtained at 100 p.p.m. concentration are summarized in table I. It should be noted that, in every case, introduction of one or more polar groups greatly reduced toxicity of otherwise comparable molecules. From a comparison of these results with those obtained with imidazoline and oxazoline derivatives, it

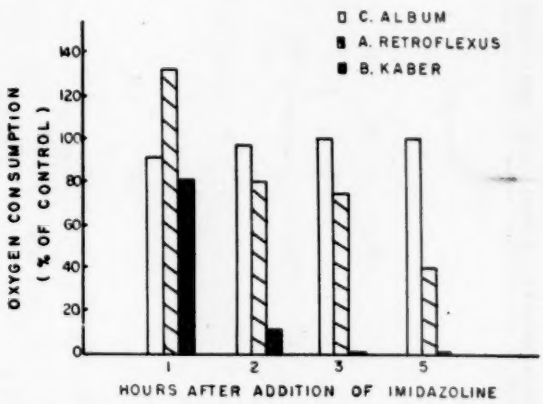


FIG. 4. The effect of 1-isopropyl-2-nonyl-4,4-dimethyl-2-imidazoline upon the respiration of leaf tissue. Imidazoline concentration 250 p.p.m. (ca. 10^{-3} Molar).

was deduced that the toxicity of the most active imidazolines was dependent upon the following structures: (a) a ring nucleus containing nitrogen and carbon atoms linked by one or more double bonds; (b) the lack of polar substituents; (c) a side chain of sufficient length to impart surface-active properties to the molecule.

RESPIRATION EXPERIMENTS.—The high toxicity and rapid action of certain imidazolines suggested that these substances have marked effects on the metabolism of plants, which might be detected by measurements of respiration. Discs of leaf tissue from young plants of wild mustard (*Brassica kaber* Wheeler), redroot pigweed (*Amaranthus retroflexus* L.) and lambsquarter (*Chenopodium album* L.) were floated in phosphate buffer (pH 7.0) in Warburg vessels maintained at 25° C. Oxygen consumption was measured for one hour before and five hours after the addition of 1-isopropyl-2-nonyl-4,4-dimethyl-2-imidazoline from the side arm. A five-step

TABLE I

INHIBITION OF SEEDLING ROOTS PRODUCED BY TREATMENT WITH A HIGHLY TOXIC IMIDAZOLINE AND CLOSELY RELATED COMPOUNDS.

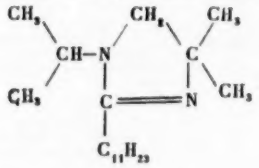
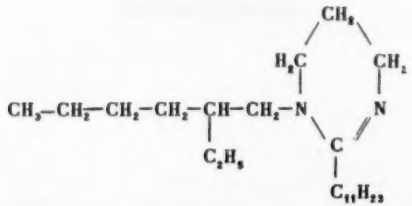
Compound	Structure	% inhibition of root growth (100 p.p.m. conc.)	
		Wheat	Radish
1-isopropyl-2-undecyl-4,4-dimethyl-2-imidazoline		95	98
1-(2-ethyl hexyl)-2-undecyl-1,4,5,6-tetrahydropyrimidine		63	69

TABLE I (*continued*)

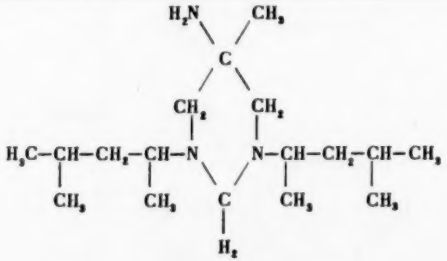
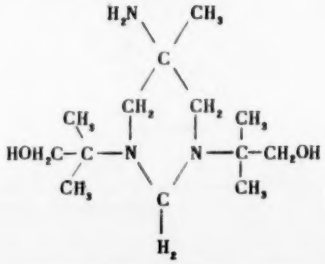
Compound	Structure	% inhibition of root growth (100 p.p.m. conc.)	
		Wheat	Radish
5-amino-1,3-bis(1,3-dimethyl butyl)-5-methyl hexahydropyrimidine		51	56
5-amino-1,3-bis(1,1,-dimethyl-2-hydroxyethyl)-5-methyl hexahydropyrimidine		0	4

TABLE I (*continued*)

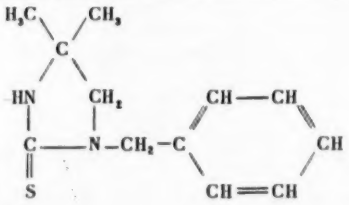
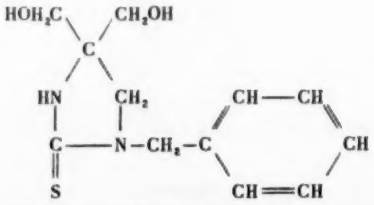
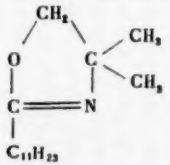
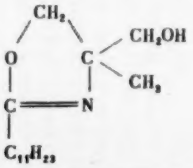
Compound	Structure	% inhibition of root growth (100 p.p.m. conc.)	
		Wheat	Radish
3-benzyl-5,5-dimethyl-2-imidazolidinethione		37	81
3-benzyl-5,5-bis(hydroxymethyl)-2-imidazolidinethione		6	0

TABLE I (*continued*)

Compound	Structure	% inhibition of root growth (100 p.p.m. conc.)	
		Wheat	Radish
2-undecyl-4,4-dimethyl-2-oxazoline		33	37
2-undecyl-4-methyl-4-hydroxy-methyl-2-oxazoline		0	19

concentration range from 0 to 500 p.p.m. of imidazoline was used and all treatments were in duplicate. The results obtained at 250 p.p.m. (c. 10^{-3} *M*) are summarized in figure 4.

Mustard tissue proved to be extremely sensitive to imidazoline, as respiration was inhibited over 90% after five hours exposure to concentrations as low as 60 p.p.m. Redroot pigweed tissue was considerably less sensitive and its oxygen consumption actually increased for the first hour after exposure to imidazoline. Lambsquarter was apparently unaffected at least during the 5-hour period of measurements.

Soil treatments.—Uniform six-inch tomato plants were treated with the three most toxic imidazolines at rates of 25, 50, 100, and 200 mg. per pot. All chemicals were dissolved in sufficient water to saturate the soil in the pots. The plants were harvested 16 days after treatment. No measurable growth differences or visible symptoms of toxicity were obtained. Previous data had shown that less than 2 mg. of imidazoline was sufficient to kill comparable tomato plants when the toxic material was sprayed on the foliage, and its addition to root systems in nutrient solutions was also toxic. It was concluded therefore that the toxic effects of imidazolines were enormously reduced as a result of contact with the soil.

Translocation of imidazolines within the plant.—Uniform tomato plants were fitted with wax paper shields to facilitate spraying particular parts without wetting other parts. When plants were sprayed on the upper half only, all treated parts were killed in a few days. With large plants, secondary buds on the untreated parts developed rapidly, as might be expected from decapitated plants. Small plants responded similarly, except that new organs were slightly malformed. This would indicate that a small amount of toxic material had been translocated downward.

With plants treated on the lower half only, all treated leaves soon abscissed and the stems became discolored and dried. However, the toxic material did not stop the functioning of conductive tissues, as the untreated upper parts continued to grow in all cases. It was therefore concluded that only a very small part of the toxic material was translocated within the tissues.

Species relationships.—The laboratory and greenhouse tests indicated that certain imidazolines might be sufficiently toxic to be of practical use as herbicides. As a survey of this possibility, preparatory to field experiments, nine species of crops and weeds were grown in the greenhouse, and young plants, four to six inches tall, were sprayed with the most toxic compounds in 0.03 to 1% concentrations. In nearly all cases, the compounds with C_9 and C_{11} chains were most toxic. The relative sensitivities of the nine species tested may be summarized as follows: (1) Resistant (minimum lethal spray concentration 0.25%): wheat (*Triticum vulgare* Vill., variety Henry), corn (*Zea mays* L., variety Wisconsin #595), lambsquarter (*Chenopodium album* L.); (2) Moderately Resistant (minimum lethal spray concentration 0.10%): soybean (*Soja max* Piper, variety Manchu), pea (*Pisum sativum* L., variety Yukon), beet (*Beta vulgaris* L., variety Detroit Dark Red), red-

root pigweed (*Amaranthus retroflexus* L.); (3) Slightly Resistant (minimum lethal spray concentration 0.05%): tomato (*Lycopersicon esculentum* Mill., variety John Baer), wild mustard (*Brassica kaber* Wheeler).

With all species, the most obvious toxicity symptoms were rapid loss of turgidity and extreme desiccation of the tissues. In a few cases, moderate formative effects were observed on plants treated with non-lethal concentrations. Additional experiments with four of the above species showed that resistance increased with age or size of the plants.

FIELD EXPERIMENTS.—A small experiment was designed to test the use of 1-isopropyl-2-nonyl-4,4-dimethyl-2-imidazoline for weed control in corn. Quadruplicate 2 by 10 hill plots of Wisconsin #595 were sprayed at two stages of growth, immediately before or 10 days after emergence of the seedlings. Imidazoline was applied at 0.5, 1 and 2 lbs. per acre in 80 gallons of water (1.6 liters per plot).

The pre-emergence treatment was made 13 days after planting, which was the last possible day before emergence of the corn seedlings. Unfavorable weather had delayed corn emergence. Weeds, mostly *Amaranthus retroflexus,* were about one inch tall at the time of the treatment. Nine days later, weed counts on all plots showed 90% control of broadleaf weeds by the 2 lbs. per acre application. Corn and grasses were unaffected.

The post-emergence treatment of weeds and corn two to six inches tall was less successful, as moderate damage was done to the corn without satisfactory control of weeds. It was concluded, therefore, that imidazolines were best adapted for control of seedling weeds before emergence of the crop plants.

A similar experiment with 4 lbs. per acre of imidazoline applied in 40, 80, and 120 gallons of water suggested that the compounds were not adapted to low-gallonage spray techniques. In addition, five by 12 foot plots of Manchu soybeans were sprayed in quadruplicate with 2, 4, and 6 lbs. per acre of 1-isopropyl-2-undecyl-4,4-dimethyl-2-imidazoline. Unfortunately, very few weeds developed on the plots prior to treatment so that the results gave primarily a measure of toxicity to the beans. Pre-emergence treatments did not cause damage but post-emergence applications caused moderate injury at all concentrations. As in the experiment with corn, grasses were relatively unaffected. It was concluded that pre-emergence treatment of soybeans might give satisfactory results, but post-emergence applications should be made with caution.

Discussion

The experiments reported here should be considered only as a basis for further experimentation. However, with the information obtained, some suggestions can be made regarding the mode of action and possible uses of the compounds. The relationships between toxicity and chain length of the substituent groups in 2-position, as well as the effects of substituent polar groups, indicate that the toxicity of the imidazolines is associated with their surface-active properties. VELDSTRA (**8, 9**) and VELDSTRA and BOOIJ (**10**)

have emphasized that surface activity is an important property contributing to the activity of plant growth substances. They point out that all active compounds contain both hydrophilic and lipophilic groups and are, therefore, active at water-lipoid interphases. They claim that the physiological activity, especially the toxicity of compounds which affect permeability, is dependent upon a delicate balance between hydrophilic and lipophilic linkages, even if other more subtle properties of the molecules are responsible for their specific growth-regulatory functions within the cell.

The results obtained with the present group of toxic compounds are in agreement at least in part with VELDSTRA's (**8**) concept. The differences in chain length requirements for imidazolines and oxazolines may be interpreted by VELDSTRA's (**9**) theory of lipophilic/hydrophilic balance. The imidazoline nucleus is more hydrophilic than the oxazoline nucleus and a slightly longer R-group is required to give the lipophilic overbalance necessary for maximum toxicity. The decreases in toxicity produced by adding polar groups to oxazolines, imidazolidinethiones and hexahydropyrimidines may also be attributed to disruption of the lipophilic/hydrophilic balance.

The above concepts would also explain the decrease in toxicity produced by lengthening the R-group to C_{17}. Such long chain compounds are so surface active that they tend to form micelles which greatly reduce the number of single molecules available for a specific reaction. Unsaturation of the long chain reduces the tendency toward micelle formation with resultant increase in toxicity.

It must be emphasized, however, that toxicity cannot be attributed solely to surface activity. Non-ionic detergents are highly surface active but are not toxic (PRILL, BARTON, and STOLT, **3**). This suggests that the imidazoline nucleus itself is toxic, but may be prevented from getting into position to react due to its hydrophilic nature. With the addition of a surface-active R-group, lipophilicity is increased so that the molecule may be absorbed by the membrane and may either react with the membrane or penetrate it to exert a toxic effect in the cytoplasm. Whether or not the effect is mainly on the surface membrane or inside the cell, the toxic imidazolines drastically affect the water-holding capacity of the tissues. When the foliage is sprayed the air spaces between the cells become filled with liquid and very rapid loss of turgor and desiccation of the affected tissue ensue.

According to PUTNAM (**4**) detergents inactivate a variety of proteins and enzyme systems; also cationic detergents, such as imidazolines, react quantitatively with proteins. This property would be expected to account at least in part for the toxicity of imidazolines and may be responsible for the effect on respiration. With sensitive tissue, such as wild mustard, the minimum concentration of 1-isopropyl-2-nonyl-4,4-dimethyl-2-imidazoline required for nearly complete inhibition of respiration was considerably less than the required cyanide concentration. The actions of the two compounds, however, are hardly comparable as the effect of the imidazoline is irreversible and probably unspecific.

In view of the above considerations the toxic action of imidazolines may

be summarized tentatively as follows: (1) Surface-active imidazolines are adsorbed on the cell and may prevent its normal function in regulating passage of materials into and out from the cell; (2) Portions of the adsorbed compounds may enter the cytoplasm to react with protein and may thus cause irreversible inhibition of respiratory enzymes.

Another interesting point in the greenhouse and field experiments was the complete inactivation of the imidazolines by soil contact. As the compounds are strong cations, they are probably tightly attached to the negative surfaces of soil colloids, so that effective physical binding takes place before they can reach the plant roots. Similar binding of the compounds onto the negatively charged cell surfaces, together with their rapid toxic action, may explain in part their failure to be translocated in the plant. The apparent lack of translocation both in the soil and in the plant restricts the action of imidazolines to contact effects. Although this limits their general use as herbicides, it may be an asset in pre-emergence treatments of sensitive crops which are seriously damaged by after-effects of herbicides now in use.

Summary

Certain members of a series of 10 1-isopropyl-4,4-dimethyl-2-imidazolines with alkyl groups substituted in the 2-position were found to be toxic to seedlings and larger plants.

Their toxicity is a function of the length and degree of unsaturation of the alkyl group in the 2-position. Compounds with alkyl groups of five or less carbons were only slightly toxic; those with seven, nine, and 11 were extremely toxic; and those with 17 carbon atoms in a saturated chain were only moderately toxic. The presence of one or more double bonds near to the middle of the C_{17} chain increased the toxicity of the latter compounds.

A comparable series of oxazolines was much less toxic, but showed a similar relationship between chain length and toxicity.

The substitution of one or more OH groups for H atoms in the methyl groups in the 4-position markedly reduced the toxicity of the compounds.

Related compounds, such as hexahydropyrimidines and imidazolidinethiones, also possessed toxic properties which were largely removed by the incorporation of OH groups into the molecules.

The most toxic imidazoline derivatives applied as sprays to young tomato plants were lethal in concentrations of 0.05%, whereas the least toxic ones produced no visible effects in concentrations of 1%.

The toxic imidazolines are effective respiratory inhibitors, but are poorly translocated through plant tissues, and are inactivated by contact with soil.

The toxic action of the imidazolines may be due in part to their lipophilic properties.

In greenhouse tests with nine species: corn, wheat, and lambsquarter were found to be relatively resistant; soybeans, table beets, peas, and redroot pigweed were moderately sensitive; and tomatoes and wild mustard were readily killed by low concentration spray applications.

Limited field tests suggest that imidazoline derivatives may be of practical use for contact killing of plants, particularly of seedling weeds before the emergence of crop plants.

Acknowledgment is made to the Commercial Solvents Corporation for financial support and for preparation of chemicals; especially to T. S. Carswell and Robert Harker for their interest and helpful advice in these investigations. Facilities and assistance with field plot tests were kindly provided through Professor K. P. Buchholz, Department of Agronomy.

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BRIEF PAPERS

A VIRUS TECHNIQUE USEFUL TO DIAGNOSE FOLIAR DEFICIENCIES

A. S. Costa and C. M. Franco

(WITH ONE FIGURE)

Received November 28, 1950

Plants of *Coffea dewevrei* De Wild. et Th. Durant var. excelsa Chev. growing in nursery beds in a lath house at the Instituto Agronomico, Campinas, Brazil, exhibit frequently a type of chlorosis in the leaves (**1**).

Repeated attempts were made to improve the condition of the chlorotic plants by spraying their leaves with salt solutions of various elements, including iron, zinc, manganese, magnesium, etc. None of these treatments induced recovery of the normal green color, but it was noticed that some leaves of the plants sprayed with ferrous sulphate showed a few small islands of green tissue. The examination of these green areas revealed that they usually surrounded a point at which the leaf had been injured. This observation was suggestive that the chlorosis might be the result of iron deficiency in the leaves, and that the penetration of the iron solution in the tissues occurred only in the vicinity of points where the leaf had been injured.

Since a slight injury of the leaf was apparently beneficial for the penetration of the iron solutions in the leaves of the chlorotic coffee plants, it occurred to the writers that the use of carborundum as an abrasive (**2**) and the half-leaf technique might be good methods to be employed for the diagnosis of foliar deficiencies. The results obtained with this technique in the study of the cholorosis of the coffee plant were very satisfactory and indicate that its application might be useful also in leaf deficiencies of other plants.

Material and methods

Preliminary tests with various concentrations of ferric chloride and ferrous sulphate indicated that a 1% solution of either salt gave satisfactory results. The methods of applying the solutions and the untreated controls were compared by pairs, in all possible combinations, the position of each treatment being alternately on the left or right half-leaf. The treatments were distributed at random in regard to position of the leaves on the plants.

A de Vilbiss atomizer was used to apply the solution. The half-leaf not to be sprayed was protected with paper while the other half-leaf was sprayed. Rubbing was done in the manner common in virus studies, using a square pad of muslin cloth moistened in the solution to rub the half-leaf. When carborundum powder (600 mesh) was used, it was sprinkled on the leaf prior to rubbing. Care was exercised when rubbing to avoid damage

to the leaf. The treatments were usually applied in the following order: spraying, rubbing, and rubbing with carborundum.

The reaction shown by the treated half-leaves and controls was graded in four classes according to degree of greening: no reaction, slight, medium, and strong reactions.

Experimental results

The results obtained in various tests comparing three methods of applying iron solutions to chlorotic leaves of the coffee plant are presented in table I. They indicate that the application of iron solutions with the atomizer was ineffective. Rubbing alone also gave poor results, whereas the use of carborundum greatly increased the proportion of half-leaves that turned green, and also the degree of the reaction (fig. 1). The greening of the treated half-leaves of the coffee plants was a slow process, taking from 10 to 30 days to become apparent. The effect of the treatment remained restricted

TABLE I

COMPARISON OF METHODS OF APPLYING SOLUTIONS OF IRON SALTS ON HALF-LEAVES OF CHLOROTIC COFFEE PLANTS.

Method of application	Half-leaves				
	No. tested	Degree of greening shown			
		None	Slight	Medium	Strong
		No.	No.	No.	No.
Spraying	66	63	3	0	0
Rubbing	46	24	15	5	2
Rubbing with carborundum	135	2	9	43	81
Untreated control*	85	84	1	0	0

* In some tests the control half-leaves were rubbed with water or with salt solutions, carborundum also being used.

to the areas actually rubbed, thus indicating that the iron solution that had penetrated in the leaf reached the underlying tissues, but did not move laterally.

In some tests a comparison was made between the use of carborundum, followed by rubbing, on the upper side versus the under side of the half-leaves. The final results were apparently alike, but there was a slight indication that the application to the upper side of the leaf induced a quicker response.

Discussion

Spraying iron salts on chlorotic leaves of coffee plants induced practically no response, whereas the application of the same solutions followed by rubbing with carborundum resulted in a strong greening of the treated half-leaves. The use of the abrasive with water or solutions of other salts did not cause any reaction. These facts indicate that the penetration of

the iron solution was greatly favored by the slight mechanical injury caused by carborundum which probably permitted the penetration of the solution into the epidermal cells, followed by distribution to the underlying palisade cells of the leaf.

It is not known whether the application of solutions by rubbing with the help of carborundum will be more effective than spraying in the case of other plants. In preliminary tests not described in this paper, this technique was useful to diagnose deficiencies shown by citrus leaves. It is to be expected that the use of an abrasive will be more advantageous in tests

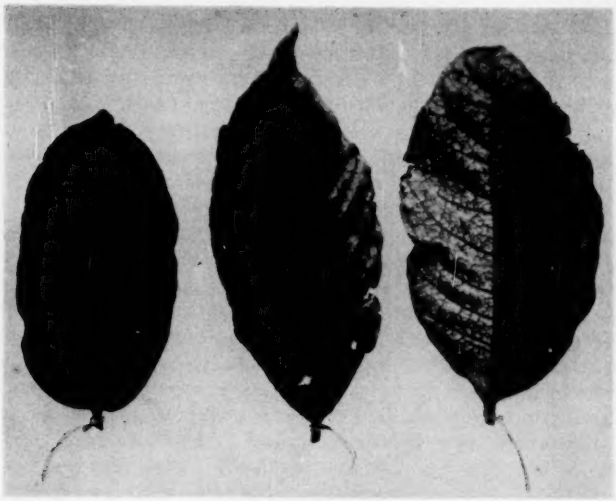

FIG. 1. Leaves of *Coffea dewevrei* var. excelsa from plants showing iron chlorosis, 30 days after the half-leaves had been treated with a 1% solution of ferrous sulphate. (a) By spraying. (b) By rubbing with carborundum.

diagnosing deficiencies in plants which possess a more impermeable cuticle, but the method might promote a quicker response to treatment even from plants which react to spray applications.

The results obtained with chlorotic coffee plants suggest that the application of iron salts, or perhaps salts of other elements on the leaves of orchard plants to correct symptoms of deficiency might be greatly helped if spraying is done with high pressure, by mixing an abrasive in the solution to be sprayed, or by other methods aimed at overcoming the resistance offered by the leaf against penetration of the solutions.

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SOME EFFECTS OF WATER AND OXYGEN ON ABSCISSION *IN VITRO*

H. R. Carns,[1] F. T. Addicott and R. S. Lynch

(WITH TWO FIGURES)

Received February 1, 1951

Some early experiments conducted in this laboratory showed an inhibition of abscission in explants (excised foliar abscission zones) immersed in water (**3**). Later experiments showed a similar inhibition in explants immersed in mineral oil or in nitrogen. Other experiments showed a retardation of abscission in explants treated daily with single 0.005 ml. droplets of water. The data from 10 such experiments with water droplets, each involving 20 or more explants of the Black Valentine bean, are summarized in figure 1. The methods employed in these experiments have already been reported (**1**).

A factor common to these experiments is restriction of the supply of oxygen. To obtain further evidence on the possibility of a relationship between restriction of the supply of oxygen and inhibition of abscission, another series of experiments was performed with explants of the Black Valentine bean. In some of the experiments explants were immersd in double distilled water in a glass-sintered filter and the gas mixture passed upward through the filter disc into the water at a rate of 100 ml. per minute. In other experiments the explants were impaled on glass pins in Petri dishes (**1**), placed in desiccators and the atmosphere of the desiccator replaced by flushing with a gas mixture at a rate of 243 ml. per minute for 80 minutes. By the end of this time only 0.1% of the original atmosphere remained. The desiccator was then sealed. Seven gas mixtures were used: 100, 60, 40, 20, 10, 5, and 0% oxygen, the balance in each case being made up of nitrogen. The mixtures were obtained by the use of the flowmeters described by Biale (**2**).

The explants in desiccators with gas mixtures ranging from 10 to 40% oxygen abscised at a rate that was positively correlated with oxygen level. The explants immersed in water through which bubbled gas mixtures ranging from 20 to 55% also abscised at a rate positively correlated with oxygen level. That is, within the limits designated, an increase in rate of abscission occurred with each increase in oxygen level. Above the oxygen levels of 40 and 55% there was, however, no further increase in rate of abscission. And at any given oxygen level below 55% the explants in water had a lower rate of abscission than did the explants in the desiccators.

These results are summarized in figure 2, where the rate of abscission at each oxygen level is expressed in the number of days required for the abscission of half the explants exposed to that level. Each point on the curve is an average of the response of from 20 to 90 explants.

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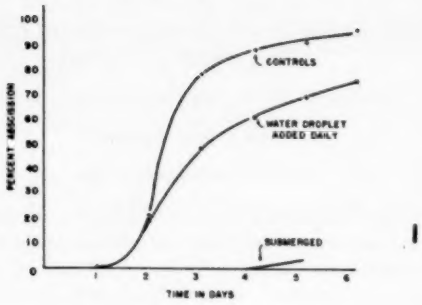

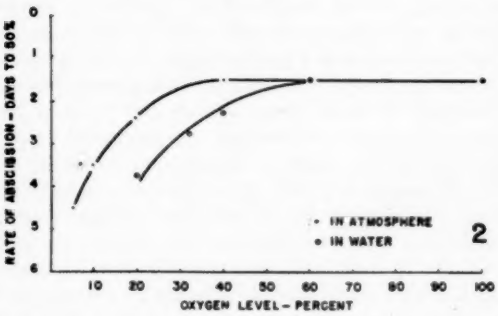

FIG. 1. The effect of water on the abscission of explants.

FIG. 2. The effect of oxygen on abscission of explants (a) in gas mixtures, (b) submerged in water.

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EXPANSION OF *CHENOPODIUM ALBUM* LEAF DISKS AS AFFECTED BY COUMARIN[1]

CARLOS O. MILLER AND BERNARD S. MEYER

(WITH ONE FIGURE)

Received February 15, 1951

Although leaf expansion is obviously a fundamental process in the development of plants, its physiological mechanism is little understood, and has been but sparingly investigated.

It is known that auxins affect the elongation of the leaf veins (**1**) but do not seem to influence the expansion of the laminar leaf tissues. BONNER *et al.* (**3**) observed an increase in expansion of radish leaf disks floated on solutions of adenine. BONNER (**2**) also found potassium nitrate to be effective in promoting expansion and (private communication) believes the salt to have both a potassium and a nitrate effect. de Ropp found several substances, including adenine, to be without effect on elongation of first leaves of isolated rye stem tips (**4**), and several substances to be without effect on the final dry and wet weights of cabbage leaf disks (**5**). Various extracts and solutions injected into the hollow petioles of squash plants did not increase the expansion of the leaves in the dark (**7**).

In an effort to achieve a better understanding of the physiology of leaf expansion, a number of growth inhibitors have been tested. Coumarin is one of these compounds. Coumarin affects Avena root elongation (**6**), elongation of Avena coleoptile sections, and curvature of split pea stem internodes (**8**). In the papers cited, emphasis has been placed on growth inhibition by the compound. However, in the latter work, increases in elongation of Avena coleoptile sections were obtained at concentrations lower than 10^{-4} M (10^{-4} M is approximately equal to 15 p.p.m.). In the work reported here, coumarin in concentrations ranging from 1 to 200 p.p.m. has been found to increase expansion of leaf disks taken from *Chenopodium album* plants growing outdoors during July, August, and September, 1950.

Disks, 5.0 mm. in diameter, were cut with a cork borer from immature leaves having areas of approximately 250 sq. mm. The largest leaves on the plants were about eight times this size. Two disks were cut from each leaf, one from the basal section on each side of the midvein. A segment of a main lateral vein constituted a diameter in each disk. Sixteen disks with lower epidermis up were floated on 10 ml. of the various solutions in Petri dishes. The dishes were placed in the dark and kept at $25 \pm 1°$ C. The check medium contained 4% D-glucose by weight and KNO_3 at 0.2 M. In preliminary experiments, these concentrations were found to be optimal. The pH of each solution was 5.6. At the end of the test period, the disk diameters, perpendicular to the segments of lateral veins, were remeasured.

[1] Papers from the Department of Botany, The Ohio State University, no. 524.

The effect of coumarin at various concentrations is shown graphically in figure 1. Similar curves have been obtained in several experiments.

The increase in diameter was less at 200 than at 100 p.p.m. Probably expansion would be inhibited at higher concentrations. However, even at 200 p.p.m. "toxic" effects were noticeable, the tissues becoming soft and brown. For this reason the effects of higher concentrations were not investigated.

The expansion of the leaf disks was accelerated by coumarin at concentrations (15–200 p.p.m.) at which elongation of Avena roots (**6**), elongation of Avena coleoptile sections, and curvature of split pea stem sections (**8**) were reduced. This difference may not be significant, however, since the experimental conditions of the various workers were not identical. Also, the difference may be partially explained by the low wettability of the leaf

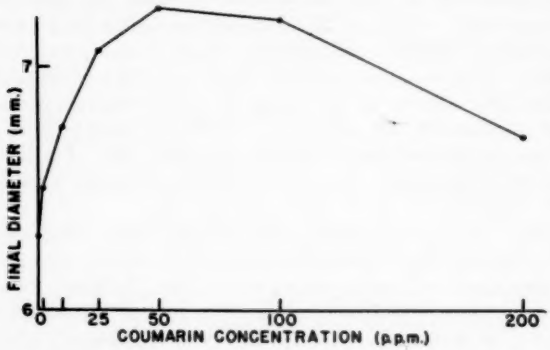

FIG. 1. Average diameters of *Chenopodium album* leaf disks after floating for two days on solutions containing 4% D-glucose, 0.2 M KNO_3, and coumarin at the concentrations indicated. The standard error for each of the first six points is ± 0.03; for the highest concentration it is ± 0.04.

disks. Thus the amount of coumarin entering the disks may have been rather small. This low wettability apparently made the disks less susceptible to attack by microörganisms and therefore more desirable as test objects than disks cut from other kinds of leaves.

A coumarin effect, although small, was found when either D-glucose or KNO_3 was omitted from the medium (table I). Apparently the action of coumarin is not to increase the permeability to either glucose or KNO_3 alone. The effect was greatest when both the salt and sugar were present. The results in table I are for one experiment only, but similar results have been obtained in two other experiments.

The D-glucose could be replaced by sucrose. KNO_3 could be at least partially replaced by KCl, $NaNO_3$, or $Mg(NO_3)_2$ but not by $Ca(NO_3)_2$.

At equivalent molar concentrations, KNO_3 was more effective than either

TABLE I

RELATIVE INCREASES IN DIAMETER OF *Chenopodium album* LEAF DISKS FLOATED ON SOLUTIONS OF VARIOUS COMBINATIONS OF 4% D-GLUCOSE, 0.2 M KNO_3, AND COUMARIN (50 P.P.M.)

Solution	Relative increase in diameter after two days
Water	100 ± 2.6*
Coumarin	105 ± 3.9
D-Glucose	117 ± 3.5
D-Glucose + coumarin	146 ± 4.4
KNO_3	146 ± 3.5
KNO_3 + coumarin	170 ± 4.9
KNO_3 + D-glucose	200 ± 6.1
KNO_3 + D-glucose + coumarin	319 ± 4.4

*Standard error.

KCl or $NaNO_3$. Thus Bonner's separation of the potassium and nitrate effects seems confirmed.

Summary

Coumarin (1–200 p.p.m.) increased the expansion of *Chenopodium album* leaf disks floating on a basic solution of D-glucose and KNO_3. A coumarin effect, although small, was found when either D-glucose or KNO_3 was omitted from the medium. The glucose could be replaced by sucrose. The KNO_3 could be at least partially replaced by KCl, $NaNO_3$, or $Mg(NO_3)_2$ but not by $Ca(NO_3)_2$. KNO_3 was more effective than either KCl or $NaNO_3$ in increasing the amount of expansion of the leaf disks.

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NOTES

New England Section.—The annual meeting of the New England section of the A. S. P. P. was held on May 4–5 at the University of Rhode Island in Kingston. Seventeen institutions were represented with sixty people coming from other states. The programs included sixteen papers in addition to a symposium on the teaching of plant physiology. The officers elected for 1951–1952 are: Chairman, Dr. F. H. Steinmetz, University of Maine; Vice-Chairman, Dr. A. W. Naylor, Yale University; Secretary-Treasurer, Dr. L. H. Jones, University of Massachusetts.

Association of Southern Agricultural Workers, Annual Convention, 1951.—The Plant Physiology Section of the Association presented a series of programs in conjunction with the annual convention held at Memphis, Tennessee, February 5–8, 1951. A symposium was held with six papers on "Isotopes in Plant Physiology Research," six papers were given in a session on the physiology of growth, and seven papers in a session on plant nutrition. Joint programs were arranged with the Soils Division and the Southern Weed Conference in which the Plant Physiology Section contributed four and two papers, respectively. A special breakfast meeting for physiologists was addressed by Dr. Paul J. Kramer of Duke University on the topic of "Causes of Injury to Plants in Flooded Soil." Abstracts of papers presented will be printed in the 1951 Proceedings of the Association.

Plant Growth Substances.—Folke Skoog, Editor. University of Wisconsin Press, 811 State Street, Madison, Wisconsin. 476 pages, 1951. $6.00.

Plant Growth Substances consists of thirty-nine papers presented during the symposium on plant growth substances at the University of Wisconsin in September 1941. The purpose of the symposium was to obtain a perspective of the present status and progress in the rapidly expanding field of control of plant growth, and the success of the symposium is demonstrated by this publication. Many of the papers present new data in addition to reviewing recent developments and outlining principal lines of investigation in the various divisions of the subject. The editor and other members of the University of Wisconsin faculty committee and the authors of the contributions have performed a very real service for students and professional workers alike. The volume fills an important need for an authoritative reference work on the control of plant growth.

The published lectures are: The History and Nature of Plant Growth Hormones by A. J. Haagen-Smit; The Synthetic Auxins—Relation Between Structure and Activity by K. V. Thimann; Growth and Structure of the Primary Wall by T. Kerr; Mechanisms of Cell Elongation by H. Burström; Control of Evolution and Life Processes in Plants by A. F. Blakeslee; Twenty Years of Plant Hormone Research by F. W. Went; and Plant Hor-

mones in Practice by P. W. Zimmerman. The remaining papers are grouped under seven headings: Growth Substances in Plant Metabolism; Tissue Responses to Growth Substances; Practical Applications of Growth Regulators; Growth Substances in Vegetative Development; Growth Substances in Reproductive Development; Growth Substances in Pathological Growth; and Vitamins and Amino Acids as Growth Factors.

Recent Advances in the Study of Plant Viruses.—K. M. SMITH. Second Edition, 1951. The Blakiston Company, Philadelphia, Penn. 300 pages, $4.50.

This publication is a revision of the first review of the field of plant viruses which appeared in 1933. The text has been entirely rewritten in an attempt to give a representative account of the large number of new developments and the great progress made in the last fifteen years. The outstanding advances in the isolation, crystallization and biochemical studies of the viruses, the serology and electron microscopy of plant viruses, and the relationship of viruses with their insect vectors constitute new material in the revision. Particularly noteworthy among the illustrations are the electron micrographs of plant viruses. The units of the book are: Symptomatology; Physiology of virus-diseased plants; Insects in relation to viruses; Methods of transmission of plant viruses; Methods of purification of viruses; Properties of purified viruses; The sizes of viruses and the methods employed in their estimation; Electron microscopy of plant viruses; Strains, mutations and variation in viruses; The serology of plant viruses; Control of plant virus diseases; and Classification and nomenclature of plant viruses. Many references are listed with each unit, and author and subject indices are included.

Scientific Russian.—J. W. PERRY. Interscience Publishers, Inc., 250 Fifth Avenue, New York 1, New York. 816 pages, 1950. $7.50.

Many plant scientists will find this publication of value. It is designed for self-study and for reference as well as for classroom instruction in a reading knowledge of scientific and technical Russian. An extensive table of contents and a subject index facilitate the use of the book as a reference. In addition to lessons in the Russian alphabet and grammar there are reading exercises, Russian-English and English-Russian vocabularies, and a list of abbreviations commonly encountered in reading Russian scientific and technical material.

Fundamentals of Horticulture.—J. B. EDMOND, A. M. MUSSER, and F. S. ANDREWS. The Blakiston Company, Philadelphia, Penn. 502 pages, 1951. $5.50.

This new textbook in horticulture emphasizes the fundamentals underlying horticultural practices. It is divided into three parts: Part I is a brief

review of plant growth and development; Part II describes the principal horticultural practices; Part III discusses the principal horticultural crops and ornamentals.

Basic Horticulture.—V. R. GARDNER. Second Edition, 1951. The Macmillan Company, 60 Fifth Avenue, New York 11, New York. 465 pages. $4.75.

This is a revised edition of the elementary textbook first published in 1942. A chapter on hormones and growth regulators and their applications in horticulture has been added, and the discussion of herbicides has been enlarged into a chapter on weeds and their control.

A.S.P.P. Election.—The results of the annual election of the American Society of Plant Physiologists for 1951–52 are as follows:

President, Dr. Cecil H. Wadleigh, U.S.D.A. Plant Industry Station, Beltsville, Maryland.

Vice-President, Dr. Theodore C. Broyer, University of California, Berkeley, California.

Secretary, Dr. Alden S. Crafts, University of California, Berkeley, California.

Member of the Executive Committee, Dr. James Bonner, California Institute of Technology, Pasadena, California.

Members of the Editorial Board: Dr. James P. Bennett, University of California, Berkeley, California; Dr. Harry A. Borthwick, U.S.D.A. Plant Industry Station, Beltsville, Maryland; Dr. Robert Emerson, University of Illinois, Urbana, Illinois; Dr. Karl C. Hamner, University of California, Los Angeles, California.

AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS

Organized in 1924 for the advancement of Plant Physiology as a pure and applied field of botanical science

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OFFICERS FOR 1951–52

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U.S.D.A. Plant Industry Station, Beltsville, Maryland

Vice-President: Dr. Theodore C. Broyer,
University of California, Berkeley, California

Secretary: Dr. Alden S. Crafts,
University of California, Davis, California

Executive Secretary-Treasurer: Dr. J. Fisher Stanfield,
Miami University, Oxford, Ohio

Any one actively interested in Plant Physiology is eligible for membership in the Society. Candidates for membership must be recommended by two members, and approved by executive committee, or by the vote of the Society at some annual or special meeting.

The payment of the annual dues to the Society entitles the members to receive the official journal, Plant Physiology. They will receive in addition the regular bulletins and announcements sent out periodically by the secretary. Members are invited to take a personal interest in the growth and development of the Society.

Manuscripts for Plant Physiology should be addressed to Prof. William F. Loomis, Department of Botany, State University of Iowa, Iowa City, Iowa. Drawings and photographs should not exceed 8.5 x 11 inches in size, should be numbered correctly on the back, and separate typewritten legends included with the text of the manuscript. Tables and cuts should be used sparingly, and the text presented in concise form. Instructions to authors for preparation of manuscripts are given in the January number, Volume 26, 1951, of Plant Physiology, pages 205–206.

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